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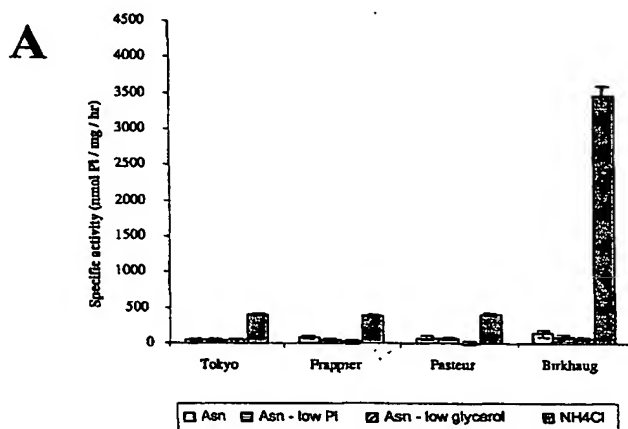
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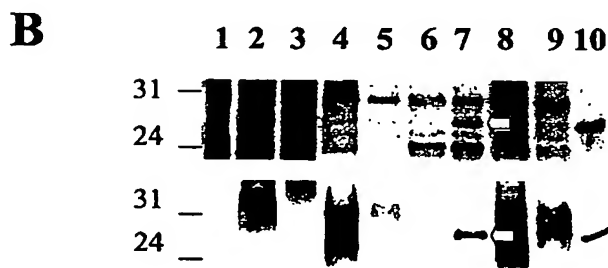
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(54) Title: SECRETED ACID PHOSPHATASE (SAPM) IS PRESENT ONLY IN PATHOGENIC MYCOBACTERIA AND EX-
RESSED SELECTIVELY AT PHAGOSOMAL PH



(57) Abstract: The invention relates to an isolated DNA construct, comprising a mycobacterium secreted acid phosphatase promoter or promoter fragment which is inducible by low pH. This invention further relates to diagnostic methods and vaccines for treatment or prophylaxis of pathogenic mycobacterial disease or infection. This invention also provides for methods of screening for compounds capable of modulating the activity, production or secretion of a mycobacterium secreted acid phosphatase.



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Secreted Acid Phosphatase (*sapM*) is Present Only in Pathogenic Mycobacteria and Expressed Selectively at Phagosomal pH

Field of invention

This invention relates to diagnostic methods and vaccines for pathogenic mycobacterial or pathogenic fungal disease or infection.

Background

Pathogenic mycobacteria including *Mycobacterium tuberculosis* reside within the phagosome of the host macrophage, a harsh environment that is detrimental to most microbes. It is evident that *M. tuberculosis* modifies this phagosomal compartment in order to enhance its own intracellular survival (Clemens and Horwitz, 1995). This includes alterations in Rab GTPase composition (Via *et al.*, 1997; Clemens *et al.*, 2000) and exclusion of the vacuolar proton ATPase (Sturgill *et al.*, 1994). Consequently, the mycobacterial phagosome is arrested at an early stage of maturation and only mildly acidified to pH 6.1 – 6.5 (Xu *et al.*, 1994; Sturgill *et al.*, 1994; Clemens and Horwitz, 1995). The ability of pathogenic mycobacteria to persist in macrophages plays a crucial role in disease establishment and progression. However, the specific bacterial factors that undermine the host processes remain largely unknown. Acting on the assumption that genes contributing to bacterial pathogenesis are differentially expressed during infection, various approaches have been used to identify *M. tuberculosis* genes whose expression are preferentially upregulated *in vivo* (i.e., within macrophages) (reviewed in (Triccas and Gicquel, 2000) or under *in vitro* conditions that mimic the host environment encountered by the bacillus (e.g., low oxygen tension, acidic pH, and nutrient starvation) (Betts *et al.*, 2002; Fisher *et al.*, 2002). Although a number of candidate virulence genes have been described, many have no known biological function and their direct roles on intracellular survival of the bacillus remain obscure. The identification of mycobacterial proteins that contribute to the replication and survival of the bacteria is critical for understanding the pathogenesis and protective mechanisms of the disease.

Intracellular bacterial pathogens including *M. tuberculosis* and *Salmonella enterica* serovar Typhimurium encounter a wide variety of environmental conditions within the host that are not accessible to commensal species. The ability to respond to environmental cues, and adjust to harsh intracellular conditions allows these pathogens to survive. Environmental cues, including temperature, osmolarity, pH, oxygen tension, and nutrient availability, have been found to act as signals for the induction of specific subsets of bacterial genes, which contributes to enhanced survival or multiplication. Many of these genes have been identified in *Salmonella*, and some have been shown to play important roles in infection and/or pathogenesis (reviewed in (Lucas and Lee, 2000). Using a variety of strategies, a number of candidate virulence genes of *M. tuberculosis* have also been identified (Triccas and Gicquel, 2000). Yet direct evidence supporting a necessary role in virulence is only available for a few genes, e.g., isocitrate lyase (*aceA*) (McKinney *et al.*, 2000), α -crystallin (*acr*) (Yuan *et al.*, 1998), and the exported repetitive protein (*erp*) (Berthet *et al.*, 1998).

It is well known that pathogenic mycobacteria actively modify the phagosomal pH. Phagosomes containing viable pathogenic *M. tuberculosis* or *M. avium* are only mildly acidified (pH 6 – 6.5), which is, at least partially, due to the paucity of proton ATPase (Sturgill *et al.*, 1994). In contrast, nonpathogenic mycobacteria such as *M. smegmatis* and *Mycobacterium goodii*, or dead pathogenic mycobacteria, do not block phagosomal acidification and the pH of phagosomes containing these bacteria is acidified to values similar to the pH of late endosomes and lysosomes (pH \approx 5) (Kuehn *et al.*, 2001). These data suggest that production of factors found only in pathogenic strains are important for inhibition of phagosomal acidification and intracellular survival. Acidification may be a signal to induce expression of genes needed to alter phagosomal maturation. SapM, the secreted acid phosphatase of *M. tuberculosis* H37Rv, was identified recently (Saleh and Belisle, 2000).

Summary

This invention provides an isolated DNA sequence comprising a promoter or promoter fragment from the 5' flanking region upstream of the start site of the

coding region of a mycobacterial secreted acid phosphatase gene [SEQ ID NO:9], [SEQ ID NO:11], [SEQ ID NO:13], [SEQ ID NO:15] wherein the promoter or promoter fragment is sufficient to control expression of a nucleotide sequence of interest and is inducible under low-pH conditions. This invention also provides an isolated DNA sequence comprising a promoter or promoter fragment from the 5' flanking region upstream of the start site of the coding region of a secreted acid phosphatase gene selected from the group consisting of *Mycobacterium tuberculosis* [SEQ ID NO:9], *Mycobacterium bovis* [SEQ ID NO:11], *Mycobacterium avium* [SEQ ID NO:13], or *Mycobacterium marinum* [SEQ ID NO:15].

The present invention relates to an isolated DNA sequence comprising a promoter or promoter fragment which hybridizes to [SEQ ID NO:1], [SEQ ID NO:2], [SEQ ID NO:3], [SEQ ID NO:4] under high stringency hybridization conditions and which is sufficient to control expression of a nucleotide sequence of interest and is inducible under low pH conditions.

The present invention also relates to an expression vector comprising an isolated DNA sequence which comprises a promoter or promoter fragment from the 5' flanking region upstream of the start site of the coding region of a mycobacterial secreted acid phosphatase gene [SEQ ID NO: 9], [SEQ ID NO:11], [SEQ ID NO:13], [SEQ ID NO:15] wherein the promoter or promoter fragment is sufficient to control expression of a nucleotide sequence of interest and is inducible under low-pH conditions.

The present invention also relates to an expression vector comprising an isolated DNA sequence which comprises a promoter or promoter fragment from the 5' flanking region upstream of the start site of the coding region of a secreted acid phosphatase gene selected from the group consisting of *Mycobacterium tuberculosis* [SEQ ID NO:9], *Mycobacterium bovis* [SEQ ID NO:11], *Mycobacterium avium* [SEQ ID NO:13], or *Mycobacterium marinum* [SEQ ID NO:15].

The present invention also relates to an expression vector comprising an isolated DNA sequence which comprises a promoter or promoter fragment which hybridizes to [SEQ ID NO:1], [SEQ ID NO:2], [SEQ ID NO:3], [SEQ ID NO:4] under high stringency hybridization conditions and which is sufficient to

control expression of a nucleotide sequence of interest and is inducible under low-pH conditions.

The invention further relates to a host cell transformed with an expression vector comprising an isolated DNA sequence which comprises a promoter or promoter fragment from the 5' flanking region upstream of the start site of the coding region of a mycobacterial secreted acid phosphatase gene [SEQ ID NO:9], [SEQ ID NO:11], [SEQ ID NO:13], [SEQ ID NO:15] wherein the promoter or promoter fragment is sufficient to control expression of a nucleotide sequence of interest and is inducible under low-pH conditions.

The invention further relates to a host cell transformed with an expression vector comprising an isolated DNA sequence which comprises a promoter or promoter fragment from the 5' flanking region upstream of the start site of the coding region of a secreted acid phosphatase gene selected from the group consisting of *Mycobacterium tuberculosis* [SEQ ID NO:9], *Mycobacterium bovis* [SEQ ID NO:11], *Mycobacterium avium* [SEQ ID NO:13], or *Mycobacterium marinum* [SEQ ID NO:15].

The invention further relates to a host cell transformed with an expression vector comprising an isolated DNA sequence which comprises a promoter or promoter fragment which hybridizes to [SEQ ID NO: 1], [SEQ ID NO:2], [SEQ ID NO:3], [SEQ ID NO:4] under high stringency hybridization conditions and which is sufficient to control expression of a nucleotide sequence of interest and is inducible under low-pH conditions.

Another aspect of the invention is a transcription cassette comprising a *sapM* promoter or a promoter fragment [SEQ ID NO 1:], [SEQ ID NO:2], [SEQ ID NO:3], [SEQ ID NO:4], a nucleotide sequence of interest operably linked to the *sapM* promoter or promoter fragment [SEQ ID NO :1], [SEQ ID NO:2], [SEQ ID NO:3], [SEQ ID NO:4] and a transcriptional termination region. In one embodiment the transcription cassette further comprises a mycobacterial secreted acid phosphatase N-terminal signal sequence [SEQ ID NO:5], [SEQ ID NO:6], [SEQ ID NO:7], [SEQ ID NO:8] or a functional portion thereof. In another embodiment the transcription cassette further comprises the secreted acid phosphatase N-terminal signal sequence selected from the group consisting of *Mycobacterium tuberculosis* [SEQ ID NO:5], *Mycobacterium*

bovis [SEQ ID NO:6], *Mycobacterium avium* [SEQ ID NO:7], or *Mycobacterium marinum* [SEQ ID NO:8].

In yet another aspect of the invention there is a method for the diagnosis of a pathogenic mycobacterial infection or a pathogenic fungal infection in a subject comprising obtaining a biological sample from a subject and analyzing the sample for the presence of antibodies specific to SapM [SEQ ID NO: 10], [SEQ ID NO:12], [SEQ ID NO:14], [SEQ ID NO:16] wherein detection of antibodies specific to SapM [SEQ ID NO:10], [SEQ ID NO:12], [SEQ ID NO:14], [SEQ ID NO:16] is indicative of the presence of the pathogenic mycobacterial infection or the pathogenic fungal infection.

In one aspect of the invention there is a method for the diagnosis of a pathogenic mycobacterial infection or a pathogenic fungal infection in a subject comprising obtaining a nucleic acid sample from a subject and analyzing the sample for the presence of nucleic acid encoding *sapM* [SEQ ID NO:9], [SEQ ID NO:11], [SEQ ID NO:13], [SEQ ID NO:15] wherein detection of *sapM* [SEQ ID NO:9], [SEQ ID NO:11], [SEQ ID NO:13], [SEQ ID NO:15] is indicative of the presence of the pathogenic mycobacterial infection or the pathogenic fungal infection. In one embodiment the method for the diagnosis of a pathogenic mycobacterial infection or a pathogenic fungal infection further comprises the step of quantifying the amount of *sapM* [SEQ ID NO: 9], [SEQ ID NO:11], [SEQ ID NO:13], [SEQ ID NO:15] detected.

Another aspect of the invention is a method for the diagnosis of a pathogenic mycobacterial infection or a pathogenic fungal infection in a subject comprising obtaining a biological sample from a subject and analyzing the sample for the presence of SapM phosphatase activity wherein detection of SapM phosphatase activity is indicative of the presence of the pathogenic mycobacterial infection or the pathogenic fungal infection.

The invention also relates to a method of screening for a compound capable of modulating or regulating production of SapM [SEQ ID NO:10], [SEQ ID NO:12], [SEQ ID NO:14], [SEQ ID NO:16] comprising providing or employing a nucleic acid construct comprising the *sapM* promoter [SEQ ID NO:1], [SEQ ID NO:2], [SEQ ID NO:3], [SEQ ID NO:4] or a functional part thereof, the promoter or the functional part thereof being operably linked to a reporter gene capable of producing a detectable or a measurable signal, exposing the

construct to a candidate or test compound and detecting or measuring the signal produced by the reporter gene, wherein a change in the signal produced in the presence of the candidate or test compound as compared to in the absence of the test compound indicates that the test compound is capable of modulating production of the mycobacterial secreted acid phosphatase. In one embodiment a plurality of compounds is tested.

The invention also relates to a method of screening for a compound capable of modulating or regulating SapM phosphatase activity comprising incubating a mixture comprising SapM [SEQ ID NO:10], [SEQ ID NO:12], [SEQ ID NO:14], [SEQ ID NO:16] a SapM substrate, and the compound to be tested, and measuring the phosphatase activity, wherein a change in activity in the presence of the test compound as compared to in the absence of the test compound indicates that the test compound is capable of modulating the activity of the secreted acid phosphatase. In one embodiment a plurality of compounds is tested.

The invention further relates to a method of screening for a compound capable of regulating or modulating SapM [SEQ ID NO:10], [SEQ ID NO:12], [SEQ ID NO:14], [SEQ ID NO:16] secretion comprising exposing mycobacterial cells to a compound to be tested, wherein the mycobacterial cells secrete SapM [SEQ ID NO:10], [SEQ ID NO:12], [SEQ ID NO:14], [SEQ ID NO:16] detecting the presence or activity of SapM [SEQ ID NO:10], [SEQ ID NO:12], [SEQ ID NO:14], [SEQ ID NO:16] secreted by the mycobacterial cells, and measuring the amount of the mycobacterial secreted acid phosphatase secreted by the mycobacterial cells, wherein a change in the secretion of the mycobacterial secreted acid phosphatase in the presence of the test compound as compared to in the absence of the test compound indicates that the test compound is capable of modulation of the secretion of the mycobacterial secreted phosphatase. In one embodiment a plurality of compounds is tested.

The invention also relates to a kit for the detection of a pathogenic mycobacterial disease or infection comprising a SapM protein [SEQ ID NO:10], [SEQ ID NO:12], [SEQ ID NO:14], [SEQ ID NO:16] or polypeptide thereof, at least one SapM antibody, and one or more reagents necessary for detection of SapM specific antibodies.

The invention also relates to a kit for the detection of a pathogenic mycobacterial disease or infection comprising an oligonucleotide comprising contiguous nucleotides from the nucleic acid sequence that is complementary to the sequence of [SEQ ID NO:1], [SEQ ID NO:2], [SEQ ID NO:3], [SEQ ID NO:4], [SEQ ID NO:9], [SEQ ID NO:11], [SEQ ID NO:13], [SEQ ID NO:15] and capable of specifically hybridizing to the complementary nucleotide sequence and reagents for hybridization of the oligonucleotide to a complementary nucleic acid sequence.

In another aspect of the invention there is an antibody which is capable of specifically binding to a SapM protein [SEQ ID NO:10], [SEQ ID NO:12], [SEQ ID NO:14], [SEQ ID NO:16] or a polypeptide fragment thereof [SEQ ID NO:22].

In yet another aspect of the invention there is a vaccine or immunogenic composition for treatment or prophylaxis of a mammal against challenge by a mycobacterium comprising an antibody which is capable of specifically binding to a SapM protein [SEQ ID NO:10], [SEQ ID NO:12], [SEQ ID NO:14], [SEQ ID NO:16] or a polypeptide fragment thereof [SEQ ID NO:22].

In a further aspect of the invention there is a vaccine or immunogenic composition for treatment or prophylaxis of a mammal against challenge by a mycobacterium comprising an isolated DNA sequence comprising a promoter or promoter fragment from the 5' flanking region upstream of the start site of the coding region of a mycobacterial secreted acid phosphatase gene [SEQ ID NO:1], [SEQ ID NO:2], [SEQ ID NO:3], [SEQ ID NO:4] wherein the promoter or promoter fragment is sufficient to control expression of a nucleotide sequence of interest and is inducible under low-pH conditions.

Another aspect of the invention relates to a vaccine or immunogenic composition for treatment or prophylaxis of a mammal against challenge by a mycobacterium comprising an isolated DNA sequence comprising a promoter or promoter fragment which hybridizes to [SEQ ID NO:1], [SEQ ID NO:2], [SEQ ID NO:3], [SEQ ID NO:4] under high stringency hybridization conditions and which is sufficient to control expression of a nucleotide sequence of interest and is inducible under low pH conditions.

The invention also relates to a vaccine or immunogenic composition for the treatment or prophylaxis of a mammal against challenge by a mycobacterium

comprising a SapM protein [SEQ ID NO:10], [SEQ ID NO:12], [SEQ ID NO:14], [SEQ ID NO:16] or a polypeptide fragment thereof [SEQ ID NO:22]. Another aspect of the invention relates to a vaccine or immunogenic composition for treatment or prophylaxis of a mammal against challenge by a mycobacterium comprising an isolated DNA sequence or sequence fragment which hybridizes to [SEQ ID NO:9], [SEQ ID NO:11], [SEQ ID NO:13], [SEQ ID NO:15] under high stringency hybridization conditions and which allows for expression of a SapM protein [SEQ ID NO:10], [SEQ ID NO:12], [SEQ ID NO:14], [SEQ ID NO:16] or a polypeptide fragment thereof [SEQ ID NO:22]. The invention further relates to an antigenic composition useful for the detection of a pathogenic mycobacterial disease or infection in a subject comprising a SapM polypeptide and substantially free from other proteins or glycoproteins with which it is natively admixed in a culture of pathogenic mycobacteria.

Detailed Description of the Drawings

Preferred embodiments of the invention will be described in relation to the drawings in which:

Figure 1. (A) Acid phosphatase activity in the culture supernatant of *M. bovis* BCG in response to nutrient stress. BCG strains, in triplicate, were grown to early stationary phase in standard, asparagine-containing Sauton medium, low phosphate Sauton, low glycerol Sauton, or ammonium-containing Sauton, and the acid phosphatase activity in the culture supernatants were determined as described under "Experimental Procedures". (B) SapM expression in *M. bovis* BCG grown in Sauton media. Equal amounts of protein (6 µg) were loaded in each lane and visualized by silver staining (top panel) or by Western blotting with SapM antisera (lower panel). Lanes 2, 4, 6, and 8 are from culture supernatant of BCG-Frappier, -Pasteur, -Birkhaug, and -Japan grown in normal Sauton medium, respectively. Lanes 3, 5, 7, and 9 are the corresponding strains grown in ammonium-containing Sauton medium. Lane 10 is partially purified SapM from mc²-155/pSAP, and lane 1 is molecular weight marker.

Figure 2. pH-dependent expression of SapM in *M. bovis* BCG-Birkhaug.

(A) Acid phosphatase activity determined in CFP fractions of BCG-Birkhaug cultures grown in Sauton medium in which the pH was adjusted to indicated levels by appropriate buffers. Results are from triplicate cultures. (B) The same CFP fractions as in panel (A) were subjected to SDS-PAGE analysis. Equal amounts of protein (7 µg) were loaded in each lane and visualized by silver staining (top panel) or by Western blotting with SapM antisera (lower panel). Partially purified SapM, indicated by the arrow, was included as a control. (C) Cell surface-associated acid phosphatase (open bars) and alkaline phosphatase (filled bars) activity from BCG-Birkhaug. The activities are detected from equal numbers of cell as described under "Experimental Procedures".

Figure 3. Cloning and expression of *M. tuberculosis* sapM in *M.*

smegmatis. (A) Construction of pSAP. A 3 kb *NheI* fragment of the *M. tuberculosis* genome (BAC403) was ligated to *XbaI*-linearized pMD31. The resulting plasmid, pSAP, contains *upp*, *sapM* (Rv3310) and *sapC* (Rv3311).

Restriction enzyme sites: B *Bgl* II, H *Hind*III, K *Kpn*I, V *Eco*RV, and X *Xba*I.

(B) Expression of *sapM* in *M. smegmatis*. Cells of *M. smegmatis* were grown in normal asparagine-containing Sauton (lanes 1 and 3) or ammonium-containing Sauton media (lanes 2 and 4), and the CFP fractions were subjected to SDS-PAGE and visualized by silver staining (top panel) or by Western blotting with SapM antisera (lower panel). Lanes 1, 2: mc²-155/pSAP, lanes 3, 4: mc²-155/pMD31. Arrow indicates SapM.

Figure 4. pH-induced expression of *M. tuberculosis sapM*. (A) *M. smegmatis* recombinant strain mc²-155/pSAP (filled bars) and control strain mc²-155/pMD31 (open bars) were grown in Sauton media adjusted to the indicated pH and the acid phosphatase activity in the CFP fractions were determined. (B & C) The same CFP fractions from panel A were subjected to SDS-PAGE analysis and visualized by (B) silver staining or (C) Western blotting. The top panel is from mc²-155/pMD31 and the lower panel from mc²-155/pSAP. Partially purified SapM, indicated by arrow, was included as a control.

Figure 5. (A) Construction of GFP-fusion vectors. A 2.5 kb *Eco*RV fragment of pSAP was ligated to *Eco*RV-linearized GFP-fusion vector pFPV27. The resulting pSAPC-GFP contains the entire *sapM* gene and a truncated *sapC* gene transcriptionally fused to *gfp*. Vector pSAPC-GFP was then cut with *Bgl*II. The 3.8 kb and 2.5 kb fragments, comprising *sapM*, *gfp* and the vector backbone, were re-ligated. The resulting pSAPM-GFP contains a truncated *sapM* gene transcriptionally fused to *gfp*. (B) J774 cells were infected with *M. marinum* bearing pSAPM-GFP (panels A-C), pSAPC-GFP (panels D-F), or cloning vector pFPV27 (panels G-H) and examined by microscope. *M. marinum* cells were grown in Sauton medium (pH 7.4) and J774 cells were infected with the bacteria for 24 hrs before analysis. Images were collected for each view in the DIC mode (panels A, D, and G), in the red fluorescence channel for Texas-red phalloidin (panels B, E, and H), and in the green fluorescence channel (panels C, F, and I). The intracellular bacteria are designated by arrows and extracellular bacteria are designated by arrowheads.

Figure 6. (A) Alignment of SapM homologs found in the genome database of *M. tuberculosis*, *M. bovis*, *M. avium* and *M. marinum*. Arrow indicates putative

signal sequence cleavage site. (B) Southern blot analysis. Plasmid pSAP, and chromosomal DNA isolated from in *M. bovis* BCG, *M. smegmatis*, and *M. chelonae* were digested with *EcoRI* or *EcoRV*, separated on an agarose gel, transferred to a nylon membrane, and hybridized with a radiolabeled *sapM* probe.

Figure 7. Reactivity of human sera with partially purified SapM from mc²-155/pSAP. Partially purified SapM was run on SDS-PAGE and transferred to nitrocellulose membranes. Individual lanes were cut and probed with a 1:150 dilution of human sera. Lanes: 1, rabbit SapM antisera generated against a synthetic peptide as described under "Experimental Procedures"; 2, sera from a healthy individual with a positive PPD test; 3 and 6, healthy individuals with unknown PPD reactivity; 4, patient with lymph node tuberculosis who has been on chemotherapy for 4 months; 5, patient with evident *M. avium* in sputum; 7, healthy individual who was vaccinated with BCG in 1977; 8, sera from a healthy individual who was vaccinated with BCG in 1953.

Detailed Description

The present invention will now be described more fully with reference to the accompanying drawings, in which preferred embodiments of the invention are shown. This invention may, however, be embodied in different forms and should not be construed as limited to the embodiments set forth herein. Rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art.

The term "antibody" as used herein, is meant to include both the native antibody, and biologically active derivatives of antibodies, such as, for example, Fab', F(ab').sub.2 or Fv as well as single-domain and single-chain antibodies. A biologically active derivative of an antibody retains the ability to bind antigen.

The term "isolated DNA sequence" as described herein includes DNA whether single or double stranded. The sequence is isolated and/or purified (i.e. from its natural environment), in substantially pure or homogeneous form, free or substantially free of nucleic acid or genes of the species of interest or origin other than the promoter or promoter fragment sequence. The DNA sequence according to the present invention may be wholly or partially synthetic. The term "isolated" encompasses all these possibilities.

The term "low-pH conditions" as used herein refers to mildly acidic conditions with a pH range of 5.0 to 7.0, more preferably 5.8 to 6.6, and most preferably 6.2.

The term "oligonucleotide" as described herein refers to a probe or primer that is single-stranded DNA or RNA, or analogs thereof, that has a sequence of nucleotides that includes at least 14, preferably at least 20, more preferably at least 50, contiguous bases that are the same as (or the complement of) any 14 or more contiguous bases set forth in any of SEQ ID NO:1 to SEQ ID NO:4, [SEQ ID NO:9], [SEQ ID NO:11], [SEQ ID NO:13], [SEQ ID NO:15]. Preferred regions from which to construct probes include 5' and/or 3' coding regions of SEQ ID NO: 9, [SEQ ID NO:11], [SEQ ID NO:13], [SEQ ID NO:15]. In addition, the entire cDNA encoding region of SEQ ID NO:18, or the entire sequence corresponding to SEQ ID NO: 20, may be used as a probe. Probes

may be labeled by methods well-known in the art, as described hereinafter, and used in various diagnostic kits.

The term "operably linked" as described herein means joined as part of the same nucleic acid molecule, suitably positioned and oriented for transcription to be initiated from the same promoter.

The term "promoter" as described herein refers to a sequence of nucleotides from which transcription may be initiated on DNA operably linked downstream (i.e. in the 3' direction on the sense strand of double-stranded DNA).

The term "promoter activity" as described herein refers to the ability to initiate transcription.

The term "transcription cassette" as described herein refers to a nucleic acid sequence encoding a nucleic acid that is transcribed. To facilitate transcription, nucleic acid elements such as promoters and enhancers and transcriptional termination sequences are typically included in the transcription cassette.

We have found that SapM is an important determinant of mycobacterial pathogenesis.

In contrast to many bacterial acid phosphatases, the expression of SapM is not regulated by environmental inorganic phosphate concentration. Instead, it is regulated by pH. Pathogenic mycobacteria produce high levels of SapM under mildly acidic pH, and expression is maximal at pH 6.2. The *sapM* gene is present in pathogenic mycobacteria only. The *sapM* gene is selectively expressed in host macrophages, and is important for the virulence and pathogenesis of mycobacteria including *M. tuberculosis*. The expression of *M. tuberculosis sapM* is induced by the intracellular pH, and this gene is specific to pathogenic mycobacteria. SapM possess unique enzymatic activity and is an important determinant of mycobacterial pathogenesis. Unlike other bacterial acid phosphatases, SapM exhibits unusually high activity against GTP, which may affect the cellular GTP content and subsequently the balance of active to inactive forms of Rab proteins. This is particularly relevant since SapM is a small protein (28 kD) secreted extracellularly and is likely to gain access to the cytosol of the macrophages. The importance of SapM function in mycobacterial pathogenesis is further illustrated by the remarkable finding that SapM is present in pathogenic mycobacteria but not in

nonpathogenic mycobacteria. SapM homologs are found in *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. avium* and *M. marinum*, all of which are pathogenic. In contrast, neither the SapM protein nor the *sapM* gene is detected in nonpathogenic, environmental mycobacteria such as *M. smegmatis* and *M. chelonae*.

Promoter Fragment

The present invention provides an isolated DNA sequence comprising a promoter or promoter fragment, the promoter or promoter fragment comprising a nucleotide sequence as provided in [SEQ ID NO:1], [SEQ ID NO:2], [SEQ ID NO:3], [SEQ ID NO:4]. The promoter or promoter fragment may comprise one or more fragments of the nucleotide sequence provided in [SEQ ID NO:1], [SEQ ID NO:2], [SEQ ID NO:3], [SEQ ID NO:4] sufficient to control expression of a nucleotide sequence of interest and which is inducible under low-pH conditions. The promoter or promoter fragment may comprise a sequence of nucleotides 5' to position 498 as shown in [SEQ ID NO:1] in *Mycobacterium tuberculosis* or an equivalent sequence in other pathogenic mycobacterium or pathogenic fungi.

Restriction enzyme or nucleases may be used to digest the nucleic acid followed by an appropriate assay to determine the minimal nucleotide sequence required for promoter activity.

The promoter or promoter fragment may comprise one or more sequence motifs or elements conferring developmental and/or tissue-specific regulatory control of expression or regulatory control by an exogenous substrate or environmental condition. For example, the promoter or promoter fragment may comprise a lung-specific regulatory control element or an element that is regulated by an exogenously added sugar, such as isopropyl-beta-D-thiogalactopyranoside.

The present invention extends to a promoter or promoter fragment which has a nucleotide sequence which is an allele, mutant, variant or derivative, by way of nucleotide addition, insertion, substitution or deletion. Alteration to the nucleotide sequence may be performed by techniques known to those skilled in the art. One or more alterations to a promoter or promoter fragment sequence or fragment of the present invention may increase or decrease

promoter activity, or decrease the magnitude of the effect of a compound or protein cable of modulating the activity of the promoter or the promoter fragment.

Expression Vector

The present invention also provides an expression vector comprising a promoter or promoter fragment as disclosed herein. The promoter or promoter fragments can be cloned into a variety of vectors by means that are well known in the art. Such a vector may comprise a suitably positioned restriction site or other means for insertion of a nucleic acid sequence of interest into the vector that is operably linked to the promoter or promoter fragment of the current invention. Such a vector may comprise a suitably positioned restriction site or other means for insertion of a nucleotide sequence of interest into the vector that is operably linked to the promoter or promoter fragment.

Suitable vectors can be chosen or constructed containing appropriate regulatory sequences, including the promoter sequences or fragments thereof, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate.

For use in an assay or experiment, commercially available vectors such as the pET-series (Stratagene), pPRO-series (Clontech), pTet-series (Clontech), BacPAK systems (Clontech) may be employed. For use in gene therapy, vectors such as pT-Rex, pIND, pcDNA, pVAX1, and pEF may be employed. Expression vectors are useful to provide high levels of polypeptide expression. Cell cultures transformed with the DNA sequences of the current invention are useful as research tools particularly for studies of the effects of SapM on phagosome trafficking and maturation. Cell cultures may be used in overexpression and research according to numerous techniques known in the art. For example, a cell line (either an immortalized cell culture or a primary cell culture) may be transfected with a vector containing a transcription cassette of the current invention which permits the assessment of the levels of nucleic acid of interest produced, the polypeptide, the functionality and the phenotype of the cells produced.

For further details see, for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al., 1989 Cold Spring Harbor Laboratory Press.

Procedures for introducing DNA into cells depends on the host used, but are well known, such as electroporation and calcium phosphate transformation methods.

Host Cell

A further aspect of the present invention provides a host cell containing a transcription cassette of the current invention. Particularly desirable host cells include *M. tuberculosis*, *M. marinum*, *M. bovis*, *M. bovis* BCG, *M. smegmatis*, members of the *M. avium* complex, *E. coli* (and derivatives such as *E. coli* BL21(DE3)), insect cell lines, such as Sf21, for protein overexpression, and mammalian cell lines, such as RAW, and J774. Examples of suitable genes to be expressed under control of the sapM promoter are: antigen 85 genes (fbpA, fbpB, fbpC), 19-Kd lipoprotein gene (lppo) and alpha-crystallin gene (acr).

Methods known in the art for transformation, include, but are not limited to, electroporation, rubidium chloride, calcium chloride, calcium phosphate or chloroquine transfection, viral infection, phage transduction and microinjection, and the use of cationic lipid and lipid/amino acid complexes, or of liposomes, or a large variety of other commercially available, and readily synthesized transfection adjuvants, are useful to transfer a SapM nucleic acid molecule into host cells.

Host cells are cultured in conventional nutrient media. The media may be modified as appropriate for inducing promoters, amplifying nucleic acid sequences of interest or selecting transformants. The culture conditions, such as temperature, composition and pH will be apparent. After transformation, transformants may be identified on the basis of selectable phenotype.

Method of Diagnosing

Detection of *sapM* nucleic acid, SapM protein, or SapM phosphatase activity is useful as a screening tool for the presence of a pathogenic mycobacterial or a pathogenic fungal infection or to monitor the progression of a pathogenic mycobacterial or a pathogenic fungal infection.

The present invention relates to a method for the diagnosis of a pathogenic mycobacterial infection or a pathogenic fungal infection in a subject comprising:

- a) obtaining a biological sample from a subject; and
- b) analyzing the sample for the presence of antibodies specific to SapM [SEQ ID NO:10], [SEQ ID NO:12], [SEQ ID NO:14], [SEQ ID NO:16] wherein detection of antibodies specific to SapM [SEQ ID NO:10], [SEQ ID NO:12], [SEQ ID NO:14], [SEQ ID NO:16] is indicative of the presence of the pathogenic mycobacterial or pathogenic fungal infection.

The sample may be analyzed for the presence of antibodies specific to SapM by using SapM in immunoassays wherein SapM can be utilized in liquid phase or bound to a solid phase carrier. In addition, SapM can be detectably labeled in various ways for use in immunoassays with anti-SapM antibodies. The preferred immunoassays for detecting anti-SapM antibodies using the methods of this invention include radioimmuno-assays, enzyme-linked immunosorbent assays (ELISA), or other assays known in the art, such as immunofluorescent assays, chemiluminescent assays, or bioluminescent assays.

The present invention relates to a method for the diagnosis of a pathogenic mycobacterial infection or a pathogenic fungal infection in a subject comprising:

- a) obtaining a nucleic acid sample from a subject; and
- b) analyzing the sample for the presence of nucleic acid encoding SapM, wherein detection of *sapM* is indicative of the presence of the pathogenic mycobacterial or pathogenic fungal infection.

The sample may be analyzed for the presence of nucleic acid encoding SapM by, for example, PCR analysis, DNA sequencing, SSCP analysis, or RFLP analysis.

In another embodiment the present invention relates to a method for the diagnosis of a pathogenic mycobacterial infection or a pathogenic fungal infection in a subject comprising:

- a) obtaining a biological fluid sample from a subject; and

- b) analyzing the sample for the presence of SapM phosphatase activity, wherein detection of SapM phosphatase activity is indicative of the presence of the pathogenic mycobacterial or pathogenic fungal infection.

The sample may be analyzed for the presence of SapM phosphatase activity by performing the acid phosphatase assay using GTP and/or NADPH as cofactors and *p*-nitrophenylphosphate as substrate.

Measurement of *sapM* nucleic acid, SapM protein, or SapM phosphatase activity may be used not only for the diagnosis of a pathogenic mycobacterial or a pathogenic fungal infection but also to monitor therapeutic response, assess prognosis, assess patient disease risk and to monitor the success of disease preventative interventions in patients at risk.

Other assays (as well as variations of the above assay) will be apparent from the description of this invention and techniques.

Method of Screening

The present invention also relates to a method of screening a synthetic compound or protein that regulates or modulates promoter activity of the promoter or promoter fragment DNA of the present invention. A method of identifying a synthetic compound or protein that regulates or modulates the promoter activity of the SapM promoter or promoter fragment comprises:

- a) employing or providing a nucleic acid construct comprising a functional part of the SapM promoter [SEQ ID NO:1], [SEQ ID NO:2], [SEQ ID NO:3], [SEQ ID NO:4] the functional part of the promoter being operably linked to a reporter gene capable of producing a detectable signal;
- b) exposing the nucleic acid construct to candidate compounds or proteins; and
- c) comparing the signal produced in the absence of the compound or protein tested.

The reporter gene preferably encodes an enzyme which catalyses a reaction that produces a detectable signal. Many examples are known, for example, *gfp* encoding the green fluorescent protein (and *gfp* variants encoding enhanced green, blue/cyan and yellow fluorescent proteins), DsRed encoding

the red fluorescent protein, *lacZ* encoding Beta-galactosidase, *gus* encoding Beta-glucoronidase, *luxAB* (or *luxCDABE*) encoding bacterial luciferase, *luc* encoding firefly luciferase, and *phoA* encoding alkaline phosphatase.

Those skilled in the art are well aware of a multitude of possible reporter genes and assay techniques that may be used to determine gene activity.

Any suitable reporter/assay may be used and it should be appreciated that no particular choice is essential to or a limitation of the present invention.

The reporter gene may be used in an in vitro or an in vivo expression system.

In addition to a promoter or a promoter fragment, expression generally requires the presence of a translational initiation region and transcriptional and translational termination regions.

Another embodiment of the current invention relates to a method of screening a synthetic compound or protein that regulates or modulates SapM phosphatase activity comprising:

- a) incubating a mixture comprising SapM [SEQ ID NO:10], [SEQ ID NO:12], [SEQ ID NO:14], [SEQ ID NO:16], a SapM substrate, and the compound to be tested;
- b) measuring the phosphatase activity; and
- c) comparing the phosphatase activity in the absence of the compound or protein to be tested.

The substrate may comprise pNPP, GTP or NADPH.

Another embodiment of the current invention relates to a method of screening a synthetic compound or protein that regulates or modulates SapM secretion comprising:

- a) exposing mycobacterium cells to a compound or protein to be tested, wherein the mycobacterium cells secrete SapM [SEQ ID NO:10]; [SEQ ID NO:12], [SEQ ID NO:14], [SEQ ID NO:16];
- b) detecting the presence or activity of SapM [SEQ ID NO:10], [SEQ ID NO:12], [SEQ ID NO:14], [SEQ ID NO:16] secreted by the mycobacterium cells; and
- c) comparing the secretion of SapM [SEQ ID NO:10], [SEQ ID NO:12], [SEQ ID NO:14], [SEQ ID NO:16] in the absence of the compound or protein to be tested.

"Modulation", "modulates" and "modulating" can refer to an increase in expression or activity, a decrease in expression or activity, a change in the type or kind of expression or activity present, a complete cessation of expression or activity (i.e., an absence of expression or activity), or the instigation of expression or activity. Suitable compounds that may be used include but are not limited to proteins, nucleic acids, small molecules, hormones, antibodies, peptides, antigens, cytokines, growth factors, pharmacological agents including chemotherapeutics, carcinogenics, or other cells (i.e. cell-cell contacts). Cells may also be screened for the effects of environmental or physiological factors such as radiation, action potentials, etc. on normal gene expression.

Other assays (as well as variations of the above assay) will be apparent from the description of this invention and techniques such as those disclosed in U.S. Patent Nos. 5,851,788, 5,736,337 and 5,767,075 which are incorporated by reference in their entirety. For example, the test compound levels may be either fixed or increased, and a plurality of compounds or proteins may be tested at a single time.

Kits

The invention includes a kit for detecting the presence of SapM nucleic acid molecule, comprising at least one probe of the invention. Kits may be prepared according to known techniques, for example, see patent nos. 5,851,788 and 5,750,653. The kit preferably includes reagents suitable for the hybridization of the probe to a complementary nucleic acid sequence. The invention also includes a kit for detecting the presence of SapM protein, comprising at least one anti-SapM antibody of the invention. Kits may be prepared according to known techniques, for example, see patent nos. 5,851,788 and 5,750,653.

The kit preferably includes an antibody, a medium suitable for the formation of an immunological complex between the antibody and a polypeptide recognized by the antibody and a reagent capable of detecting the immunological complex to ascertain the presence of SapM or a similar polypeptide in a biological sample. Further background on the use of

antibodies is provided, for example in U.S. Patent Nos. 5,695,931 and 5,837,472 which are incorporated by reference in their entirety.

Preparation of Antibodies

The invention includes an isolated antibody immunoreactive with a polypeptide of the invention. Antibodies are preferably generated against epitopes of native SapM [SEQ ID NO:10], [SEQ ID NO:12], [SEQ ID NO:14], [SEQ ID NO:16] or synthetic peptides of SapM [SEQ ID NO:10], [SEQ ID NO:12], [SEQ ID NO:14], [SEQ ID NO:16]. The antibody may be labeled with a detectable marker or unlabeled. The antibody is preferably a monoclonal antibody or a polyclonal antibody. SapM antibodies can be employed to screen organisms containing SapM polypeptides. The antibodies are also valuable for immuno-purification of polypeptides from crude extracts. For example, one may contact a biological sample with the antibody under conditions allowing the formation of an immunological complex between the antibody and a polypeptide recognized by the antibody and detecting the presence or absence of the immunological complex whereby the presence of SapM or a similar polypeptide is detected in the sample. The invention also includes compositions, preferably including the antibody, a medium suitable for the formation of an immunological complex between the antibody and a polypeptide recognized by the antibody and a reagent capable of detecting the immunological complex to ascertain the presence of SapM or a similar polypeptide.

To recognize SapM, one may generate antibodies against a range of unique epitopes throughout the molecule, for example [SEQ ID NO:22] (NDMHDGSI). One could generate antibodies that target the N-terminal signal sequence, to block the secretion of SapM. In addition, these antibodies, or other antibodies directed against other SapM epitopes, could block SapM activity by leading to enhanced SapM clearance/degradation, with a concomitant decrease in SapM phosphatase activity.

Monoclonal and polyclonal antibodies are prepared according to the description in this application and techniques known in the art. For examples of methods of the preparation and uses of monoclonal antibodies, see U.S. Patent Nos. 5,688,681, 5,688,657, 5,683,693, 5,667,781, 5,665,356,

5,591,628, 5,510,241, 5,503,987, 5,501,988, 5,500,345 and 5,496,705 that are incorporated by reference in their entirety. Examples of the preparation and uses of polyclonal antibodies are disclosed in U.S. Patent Nos.

5,512,282, 4,828,985, 5,225,331 and 5,124,147 which are incorporated by reference in their entirety.

The invention also includes methods of using the antibodies. For example, the invention includes a method for detecting the presence of a SapM polypeptide such as [SEQ ID NO:10], [SEQ ID NO:12], [SEQ ID NO:14], [SEQ ID NO:16] or [SEQ ID NO:22] by: a) contacting a sample containing one or more polypeptides with an antibody of the invention under conditions suitable for the binding of the antibody to polypeptides with which it is specifically reactive; b) separating unbound polypeptides from the antibody; and c) detecting antibody which remains bound to one or more of the polypeptides in the sample.

Immunogenic Compositions

One skilled in the art will appreciate that suitable methods of administering the immunogenic compositions of the present invention are available. Preferably, the compositions are administered parenterally, e.g., intravenously, intraarterially, intrathecally, subcutaneously, intradermally or intramuscularly. The requirements for effective pharmaceutical carriers for parenteral compositions are well known to those of ordinary skill in the art (see, e.g. Banker and Chalmers (eds.), *Pharmaceutics and Pharmacy Practice*, J.B. Lippincott Company, Philadelphia, PA, (1982) and Toissel, *ASHIP Handbook on Injectable Drugs* (4th ed.)). Such solutions can contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulations isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. Compounds may be administered in a physiologically acceptable diluent in pharmaceutical carrier, such as sterile liquid or mixture of liquids, including water, saline, aqueous dextrose and related sugar solutions, an alcohol, such as ethanol, isopropanol, or hexadecyl alcohol, glycols such as propylene glycol or polyethylene glycol, dimethylsulfoxide, glycerol ketals such as 2,2-dimethyl-1,3-dioxolane-4-

methanol, ethers, such as poly(ethyleneglycol) 400, an oil, a fatty acid, a fatty acid ester or glyceride or an acetylated fatty acid glyceride with or without the addition of a pharmaceutically acceptable surfactant, such as soap or a detergent, suspending agent, such as pectin, carbomers, methyl cellulose, hydroxypropylmethylcellulose or carboxymethylcellulose or emulsifying agents and other pharmaceutical adjuvants.

Oils useful in parenteral formulations include petroleum, animal, vegetable, or synthetic oils. Specific examples of oils useful in such formulations include peanut, soybean, cottonseed, corn, olive, petrolatum, and mineral. Suitable fatty acids for use in parenteral formulations include oleic acid, stearic acid, and isostearic acid. Ethyl oleate and isopropyl myristate are examples of suitable fatty acid esters.

Suitable soaps for use in parenteral formulations include fatty alkali metal, ammonium, and triethanolamine salts, and suitable detergents including, cationic detergents, anionic detergents, nonionic detergents, amphoteric detergents and mixtures thereof.

The parenteral formulations typically will contain from about 0.5 to about 25% by weight of the active ingredient in solutions. Preservatives and buffers may be used. Parenteral formulations can be presented in unit-dose or multi-dose sealed containers and can be lyophilized. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules, and tablets. Topical formulations, including those that are useful for transdermal drug release, are well-known to those of skill in the art and are suitable in the context of the present invention for application to the skin.

Formulations suitable for oral administration can consist of liquid solutions, capsules, powders, suspensions and suitable emulsions. Liquid formulations may include diluents, such as water and alcohol. Capsule forms can be of the ordinary hard- or soft-shelled gelatin type containing, for example, surfactants, lubricants, and inert fillers such as lactose, sucrose, calcium phosphate, and cornstarch. Tablet forms can include excipients, colorants, diluents, buffering agents, disintegrating agents, moistening agents, preservatives, flavoring agents and pharmacologically compatible excipients.

The immunogenic compositions of the current invention can be made into aerosol formulations to be administered by inhalation. Formulations suitable

for aerosol administration may include surfactants and propellant. A carrier may also be included for intranasal delivery.

The compositions of the present invention may also be administered as suppositories by mixing with a variety of bases, such as emulsifying bases or water-soluble bases. Formulations suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulas.

The concentration of SapM protein or polypeptide fragment thereof for immunogenic compositions can vary widely, i.e., from less than about 1% usually at or at least about 10% to as much as 20 to 50% or more by weight and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail, for example, *Remington's Pharmaceutical Science* (17th ed., Mack Publishing company, Easton, PA, (1985).

The compounds of the present invention may be formulated as inclusion complexes or liposomes. Liposomes serve to target the SapM protein or polypeptide fragment thereof peptides to a particular tissue. Liposomes can also increase the half-life of the compositions.

Vaccine Preparation and Use

Immunogenic compositions, suitable to be used as vaccines, may be prepared from SapM protein and polypeptide fragments thereof and nucleic acid molecules encoding SapM proteins and polypeptide fragments thereof. For examples of methods of the preparation and uses of vaccines, see U.S. Patent Nos. 4,601,903, 4,599,231, 4,599,230, and 4,596,792 that are incorporated by reference in their entirety.

Immunogenic compositions including vaccines may be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified, or the protein encapsulated in liposomes. The live immunogenic ingredients are often mixed with excipients that are pharmaceutically acceptable and compatible with the active

ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants that enhance the effectiveness of the vaccine. Examples of adjuvants which may be effective include but are not limited to: aluminum hydroxide, N-acetylmuramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn -glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE), and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80™ emulsion.

The effectiveness of an adjuvant may be determined by measuring the amount of antibodies directed against an immunogenic polypeptide containing a SapM antigenic sequence resulting from administration of the SapM vaccines that are also comprised of the various adjuvants.

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1%-2%. Oral formulations include such normally employed excipients as, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10%-95% of active ingredient, preferably 25%-70%. The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be prophylactically and/or therapeutically effective.

The vaccine may be given in a single dose schedule, or preferably in a multiple dose schedule. A multiple dose schedule is one in which a primary

course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reinforce the immune response, for example, at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. The dosage regimen will also, at least in part, be determined by the need of the individual and be dependent upon the judgment of the practitioner.

In addition, the vaccines may be administered in conjunction with other immunoregulatory agents, for example, immune globulins.

Nucleic Acid Molecules

Functionally equivalent nucleic acid molecule or polypeptide sequence

The term "isolated DNA sequence" refers to a DNA sequence the structure of which is not identical to that of any naturally occurring DNA sequence or to that of any fragment of a naturally occurring DNA sequence spanning more than three separate genes. The term therefore covers, for example, (a) DNA which has the sequence of part of a naturally occurring genomic DNA molecule; (b) a DNA sequence incorporated into a vector or into the genomic DNA of a prokaryote or eukaryote, respectively, in a manner such that the resulting molecule is not identical to any naturally occurring vector or genomic DNA; (c) a separate molecule such as cDNA, a genomic fragment, a fragment produced by reverse transcription of polyA RNA which can be amplified by PCR, or a restriction fragment; and (c) a recombinant DNA sequence that is part of a hybrid gene, i.e., a gene encoding a fusion protein. Specifically excluded from this definition are nucleic acids present in mixtures of (i) DNA molecules, (ii) transfected cells, and (iii) cell clones, for example, as these occur in a DNA library such as a cDNA or genomic DNA library.

Modifications in the DNA sequence, which result in production of a chemically equivalent or chemically similar amino acid sequence, are included within the scope of the invention. Modifications include substitution, insertion or deletion of nucleotides or altering the relative positions or order of nucleotides.

Variants of the polypeptides of the invention may occur naturally by mutation for example, or may be made with polypeptide engineering techniques such as site directed mutagenesis for example, which are well known in the art for

substitution of amino acids. For example, a hydrophobic residue such as alanine may be substituted with a more hydrophobic residue such as leucine, valine or isoleucine. A negatively charged amino acid such as aspartic acid may be substituted for glutamic acid. A positively charged amino acid such as lysine may be substituted for another positively charged amino acid such as arginine.

Therefore, the invention includes polypeptides having conservative changes or substitutions in amino acid sequences. Conservative substitutions insert one or more amino acids, which have similar chemical properties as the replaced amino acids. The invention includes sequences where conservative substitutions are made that do not destroy SapM activity.

The invention also includes polypeptide fragments of the polypeptides of the invention that may be used to confer SapM activity if the fragments retain activity. The invention also includes polypeptide fragments of the polypeptides of the invention which may be used as a research tool to characterize the polypeptide or its activity. Such polypeptides preferably consist of at least 5 amino acids. In preferred embodiments, they may consist of 6 to 10, 11 to 15, 16 to 25, 26 to 50, 51 to 75, 76 to 100 or 101 to 250 amino acids of the polypeptides of the invention (or longer amino acid sequences). The fragments preferably have SapM activity. Fragments may include sequences with one or more amino acids removed, for example, C-terminus amino acids in a SapM sequence.

This invention also includes sequences where conservative substitutions are made that increase or decrease the activity of SapM.

Polypeptides comprising one or more d-amino acids are contemplated within the invention. Also contemplated are polypeptides where one or more amino acids are acetylated at the N-terminus. Those of skill in the art recognize that a variety of techniques are available for constructing polypeptide mimetics with the same or similar desired SapM activity as the corresponding polypeptide compound of the invention but with more favourable activity than the polypeptide with respect to solubility, stability, and/or susceptibility to hydrolysis and proteolysis. See, for example, Morgan and Gainor, (1989) *Ann. Rep. Med. Chem.*, **24**:243-252. Examples of polypeptide mimetics are described in U.S. Patent No. 5,643,873. Other patents describing how to

make and use mimetics include, 5,786,322, 5,767,075, 5,763,571, 5,753,226, 5,683,983, 5,677,280, 5,672,584, 5,668,110, 5,654,276, 5,643,873. Mimetics of the polypeptides of the invention may also be made according to other techniques known in the art, for example, by treating a polypeptide of the invention with an agent that chemically alters a side group by converting a hydrogen group to another group such as a hydroxy or amino group.

Mimetics preferably include sequences that are either entirely made of amino acids or sequences that are hybrids including amino acids and modified amino acids or other organic molecules.

The invention also includes hybrid nucleic acid molecules and polypeptides, for example where a *sapM* DNA sequence from one species is combined with a nucleotide sequence from a sequence of plant, mammal, bacteria or yeast to encode a fusion polypeptide. The invention includes a fusion protein having at least two components, wherein a first component of the fusion protein comprises a polypeptide of the invention, preferably a full length SapM polypeptide (or a portion thereof, see below). The second component of the fusion protein preferably comprises a tag, for example GST, an epitope tag or an enzyme. The fusion protein may also comprise a histochemical or cytochemical marker such as *lacZ*, alkaline phosphatase, or horseradish peroxidase, or a fluorescent marker such as GFP or one of its derivatives.

The invention also includes a composition comprising all or part of an isolated DNA molecule (preferably *sapM* [SEQ ID NO:9], [SEQ ID NO:11], [SEQ ID NO:13], [SEQ ID NO:15]) of the invention with or without a carrier, preferably in a composition for cell transformation. The invention also includes a composition comprising an isolated SapM polypeptide (preferably SapM [SEQ ID NO:10], [SEQ ID NO:12], [SEQ ID NO:14], [SEQ ID NO:16] or [SEQ ID NO: 22]) with or without a carrier, preferably for studying or modulating polypeptide activity.

Sequence Identity

The invention includes modified nucleic acid molecules with a sequence identity at least about: >17%, >20%, >30%, >40%, >50%, >60%, >70%, >80% or >90% more preferably at least about >95%, >99.5%, to the DNA sequences provided in [SEQ ID NO: 1] to [SEQ ID NO: 4] or [SEQ ID NO:9],

[SEQ ID NO:11], [SEQ ID NO:13], [SEQ ID NO:15] (or a partial sequence thereof or their complementary sequence). Preferably about 1, 2, 3, 4, 5, 6 to 10, 10 to 25, 26 to 50, 51 to 100, or 101 to 250 nucleotides are modified.

Sequence identity is most preferably assessed by the algorithm of the BLAST version 2.1 program advanced search (parameters as above). BLAST is a series of programs that are available online at <http://www.ncbi.nlm.nih.gov/BLAST>.

References to BLAST searches are:

References to BLAST searches are:

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990) "Basic local alignment search tool." J. Mol. Biol. **215**:403-410.

Gish, W. & States, D.J. (1993) "Identification of protein coding regions by database similarity search." Nature Genet. **3**:266-272.

Madden, T.L., Tatusov, R.L. & Zhang, J. (1996) "Applications of network BLAST server" Meth. Enzymol. **266**:131-141.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D.J. (1997) "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs." Nucleic Acids Res. **25**:3389-3402.

Zhang, J. & Madden, T.L. (1997) "PowerBLAST: A new network BLAST application for interactive or automated sequence analysis and annotation." Genome Res. **7**:649-656.

The polypeptides encoded by the homologous *sapM* nucleic acid molecule in other species will have amino acid sequence identity at least about: >20%, >25%, >28%, >30%, >40% or >50% to an amino acid sequence as provided in [SEQ ID NO:10], [SEQ ID NO:12], [SEQ ID NO:14], [SEQ ID NO:16] (or a partial sequence thereof). Some species may have polypeptides with a sequence identity of at least about: >60%, >70%, >80% or >90%, more preferably at least about: >95%, >99% or >99.5% to all or part of an amino acid sequence in as shown in [SEQ ID NO:10], [SEQ ID NO:12], [SEQ ID NO:14], [SEQ ID NO:16] (or a partial sequence thereof). Identity is calculated according to methods known in the art. Sequence identity is most preferably assessed by the BLAST version 2.1 program advanced search (parameters as above). Preferably about: 1, 2, 3, 4, 5, 6 to 10, 10 to 25, 26 to 50, 51 to 100, or 101 to 250 nucleotides or amino acids are modified.

The invention includes nucleic acid molecules with mutations that cause an amino acid change in a portion of the polypeptide not involved in providing SapM activity or an amino acid change in a portion of the polypeptide involved in providing SapM activity so that the mutation increases or decreases the activity of the polypeptide.

The sequences of the invention can be prepared according to numerous techniques. The invention is not limited to any particular preparation means. For example, the nucleic acid molecules of the invention can be produced by cDNA cloning, genomic cloning, cDNA synthesis, polymerase chain reaction (PCR) or a combination of these approaches (Current Protocols in Molecular Biology, F.M. Ausbel et al., 1989). Sequences may be synthesized using well-known methods and equipment, such as automated synthesizers.

Hybridization

Other functional equivalent forms of the SapM DNA molecules can be isolated using conventional DNA-DNA or DNA-RNA hybridization techniques. These nucleic acid molecules and the SapM sequences can be modified without significantly affecting their activity.

The present invention also includes nucleic acid molecules that hybridize to one or more of the DNA sequences provided in [SEQ ID NO:1] to [SEQ ID NO:4] and [SEQ ID NO:9], [SEQ ID NO:11], [SEQ ID NO:13], [SEQ ID NO:15] (or a partial sequence thereof or their complementary sequence) and that encode peptides or polypeptides exhibiting substantially equivalent activity as that of a SapM polypeptide produced by the DNA in [SEQ ID NO:10], [SEQ ID NO:12], [SEQ ID NO:14], [SEQ ID NO:16]. Such nucleic acid molecules preferably hybridize to all or a portion of *sapM* or its complement under low, moderate (intermediate), or high stringency conditions as defined herein (see Sambrook et al. (most recent edition) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; Ausubel et al. (eds.), 1995, Current Protocols in Molecular Biology, (John Wiley & Sons, NY)). The portion of the hybridizing nucleic acids is typically at least 15 (e.g. 20, 25, 30 or 50) nucleotides in length. The hybridizing portion of the hybridizing nucleic acid is at least 80% e.g. at least 95% or at least 98% identical to the sequence or a portion or all of a nucleic

acid encoding a SapM polypeptide, or its complement. Hybridizing nucleic acids of the type described herein can be used, for example, as a cloning probe, a primer (e.g. a PCR primer) or a diagnostic probe. Hybridization of the oligonucleotide probe to a nucleic acid sample typically is performed under stringent conditions. Nucleic acid duplex or hybrid stability is expressed as the melting temperature or T_m , which is the temperature at which a probe dissociates from a target DNA. This melting temperature is used to define the required stringency conditions. If sequences are to be identified that are related and substantially identical to the probe, rather than identical, then it is useful to first establish the lowest temperature at which only homologous hybridization occurs with a particular concentration of salt (e.g. SSC or SSPE). Then, assuming that 1% mismatching results in a 1 degree Celsius decrease in the T_m , the temperature of the final wash in the hybridization reaction is reduced accordingly (for example, if sequences having greater than 95% identity with the probe are sought, the final wash temperature is decreased by 5 degrees Celsius). In practice, the change in T_m can be between 0.5 degrees Celsius and 1.5 degrees Celsius per 1% mismatch. Low stringency conditions involve hybridizing at about: 1XSSC, 0.1% SDS at 50°C. High stringency conditions are: 0.1XSSC, 0.1% SDS at 65°C. Moderate stringency is about 1X SSC, 0.1% SDS at 60 degrees Celsius. The parameters of salt concentration and temperature can be varied to achieve the optimal level of identity between the probe and the target nucleic acid. The present invention also includes nucleic acid molecules from any source, whether modified or not, that hybridize to genomic DNA, cDNA, or synthetic DNA molecules that encode the amino acid sequence of a SapM polypeptide, or genetically degenerate forms, under salt and temperature conditions equivalent to those described in this application, and that code for a peptide, or polypeptide that has SapM activity. Preferably the polypeptide has the same or similar activity as that of a SapM polypeptide. A nucleic acid molecule described above is considered to be functionally equivalent to a *sapM* nucleic acid molecule of the present invention if the polypeptide encoded by the nucleic acid molecule is recognized in a specific manner by a SapM-specific antibody, including, but not restricted to, the antibodies listed in this application.

Expression of *sapM* is induced in *M. bovis* BCG grown on ammonium chloride

Bacterial acid and alkaline phosphatases are typically regulated by environmental inorganic phosphate concentration (reviewed in (Rossolini *et al.*, 1998)). The expression of these enzymes, which catalyze the hydrolysis of exogenous sources of phosphorylated components into inorganic phosphate, is induced under phosphate starvation. This essential limiting nutrient is then transported into the cell by membrane permeases. A recent study showed that SapM of *M. tuberculosis* is an acid phosphatase secreted to the culture medium (Saleh and Belisle, 2000). To determine if SapM is involved in phosphate assimilation, the effect of environmental phosphate concentration on SapM expression and activity were examined. Four *M. bovis* BCG strains, organisms closely related to *M. tuberculosis*, were grown in Sauton medium containing three different concentrations of inorganic phosphate, 0.05, 0.5, and 5.0 g/L, and the acid phosphatase activity in the culture filtrate protein (CFP) fractions was assayed. Under these conditions, acid phosphatase activity is low, ranging from 40- to 130 -nmol·hr⁻¹·mg⁻¹ (total protein). In contrast to most bacterial acid phosphatases, the acid phosphatase activity in the CFP fraction of BCG cultures was not regulated by the phosphate concentration, and phosphate starvation did not increase its activity (Fig. 1A). Starvation of carbon source in the culture medium also did not affect the enzyme activity (Fig. 1A).

Interestingly, high levels of acid phosphatase activity were detected in BCG grown in media containing ammonium chloride (NH₄Cl) as the nitrogen source (Fig. 1A). The original Sauton medium contains 27 mM of asparagine as the primary nitrogen source. Asparagine was substituted by NH₄Cl at the equal molar concentration and the fresh medium was adjusted to pH 7.4. All BCG strains tested, BCG-Birkhaug, -Japan, -Frappier and -Pasteur, grow normally in the ammonium-containing Sauton medium. The CFP fractions prepared under these conditions exhibited much higher levels of acid phosphatase activity than those grown in the original Sauton (Fig. 1A). BCG-Birkhaug exhibited a dramatic 25-fold increase, from 136- to 3,459- nmol·hr⁻¹·mg⁻¹. Other BCG strains exhibited 5- to 10-fold increases of acid phosphatase

activity. Adjustment of phosphate or glycerol concentrations in the ammonium-containing Sauton media did not change the enzyme activity level (data not shown).

It was previously shown that SapM is the only acid phosphatase detected in the extracellular fraction of *M. tuberculosis* cultures (Saleh and Belisle, 2000). To determine if SapM contributes to the observed increase of acid phosphatase activity, CFP fractions were analyzed by SDS-PAGE. As shown in Fig. 1B, a protein band, visualized by silver staining, was produced by BCG-Birkhaug grown in ammonium-containing Sauton, and was not detected in culture grown in normal Sauton medium. The mobility of this protein corresponds to the molecular weight of the mature SapM protein (~28 kD) and to that of partially purified SapM. To confirm the identity of this protein, an antibody raised against a synthetic peptide corresponding to amino acid residues 201–208 of SapM protein was made and Western blotting performed. Indeed, the 28-kD protein reacted with the anti-SapM antisera, indicating that this protein is SapM (Fig. 1B). For other BCG strains, SapM protein is less visible, which correlates with the lower level of enzyme activity detected in these strains (Fig. 1A).

SapM of *M. bovis* BCG is selectively expressed at mildly acidic pH

It was not immediately clear why SapM expression would be induced in media containing NH_4Cl . Although our initial hypothesis was that *sapM* was regulated by the availability of nitrogen source, nitrogen starvation did not alter the SapM expression or activity (data not shown). However, uptake of ammonia (NH_4^+) is accomplished by a membrane transporter, which may couple ammonia uptake to H^+ export (Westhoff *et al.*, 2002). This would result in a gradual acidification of the culture medium. Indeed, measuring the pH of the spent BCG culture media revealed that cell growth leads to acidification of Sauton medium containing NH_4Cl (i.e., the pH changed from 7.4 to 5.8 – 6.2), whereas the pH of asparagine-containing Sauton medium remains at 7.4 – 7.8.

We therefore hypothesized that the acidification of the culture medium is responsible for the induction of SapM expression. To test this, BCG-Birkhaug was grown in asparagine-containing Sauton medium (pH 7.4), then portions of cells were washed and cultured in the same medium except the pH was adjusted to 7.0, 6.6, 6.2, and 5.8 with appropriate buffers. Acid phosphatase activity from the CFP fractions of these cultures was determined and revealed a correlation between the level of acid phosphatase activity and the pH of the culture media (Fig. 2A). At pH 7.0 – 7.4, the level of acid phosphatase activity is very low. Under mildly acidic conditions (i.e., pH 5.8 – 6.6), a high level of acid phosphatase activity was detected and was maximal at pH 6.2 (Fig. 2A). SDS-PAGE and Western blotting analyses confirmed that SapM expression is induced at pH 5.8 – 6.6 (Fig. 2B), which correlates well with the high levels of enzyme activity at the same pH. These results indicate that *sapM* is selectively expressed at mildly acidic pH. The level of SapM activity detected in BCG-Birkhaug grown at pH 5.8 – 6.2 is similar to that grown in ammonium-containing Sauton medium (compare Figs. 1A & 2A), suggesting that the induction of SapM expression by ammonium described above actually reflects the effect of pH.

At pH 7.0 – 7.4, SapM was not detected either by silver staining or Western blotting (Fig. 2B), suggesting that SapM level is very low or below detection limits, which is consistent with the residual acid phosphatase activity detected at these pH values (Fig. 2A). Alternatively, the residual acid phosphatase

activity may originate from a small fraction of cell surface-associated acid phosphatase that leaks to the external medium during cell growth. Other than SapM, only one gene product of *M. tuberculosis*, Rv2577, is predicted to be an acid phosphatase (Cole *et al.*, 1998). However, Rv2577 does not possess an export signal sequence and appears to be cell surface associated (Braibant and Content, 2001). The activities of cell surface-associated acid phosphatase and alkaline phosphatase of BCG were not affected by the change of pH in the culture medium (Fig. 2C), suggesting that the pH-dependent expression is a specific property of SapM.

Cloning and expression of *M. tuberculosis sapM* in *Mycobacterium smegmatis*

SapM was originally identified by biochemical approaches from the extracellular medium of *M. tuberculosis* H37Rv grown in glycerol-alanine-salts (GAS) medium (Saleh and Belisle, 2000). Like our ammonium-containing Sauton medium, GAS contains NH₄Cl as a nitrogen source and the spent GAS medium is mildly acidic (data not shown). To confirm that the SapM of *M. tuberculosis* shares the same property as that of *M. bovis* BCG, a DNA fragment containing the *sapM* gene (Rv3310) was cloned into shuttle vector pMD31 to generate pSAP (Fig. 3A), which was then transformed into *Mycobacterium smegmatis* mc²-155. The *sapM* gene is absent from the *M. smegmatis* chromosome and SapM activity was not detected in the extracellular CFP fraction (see below). However, when recombinant *M. smegmatis* containing *sapM* was grown in Sauton medium containing NH₄Cl, SapM protein was detected upon SDS-PAGE and Western blotting analyses of the CFP fraction (Fig. 3B). SapM was absent from the pSAP strain grown in Sauton containing asparagine, and from the control strain containing the cloning vector pMD31. These results indicated that *M. tuberculosis sapM* gene can be expressed and secreted from *M. smegmatis*.

To examine if *M. tuberculosis sapM* is regulated by pH, mc²-155/pSAP was grown in Sauton medium (containing asparagine) adjusted to various pH levels, as described above. The enzyme activity and the protein concentration of SapM correlated with each other, and both were dependent on the pH of the culture medium (Figs. 4A - C). High levels of SapM expression and activity

were detected at pH 5.8 – 6.6, which is in agreement with results obtained in *M. bovis* BCG. The control strain containing the cloning vector alone showed residual acid phosphatase activity, which is likely due to leakage of the cell surface-associated acid phosphatase, and is not pH inducible.

The *M. tuberculosis sapM* gene is selectively expressed inside macrophages

Our *in vitro* studies demonstrate that *sapM* is selectively expressed at pH 5.8 – 6.6, a pH level that coincides with mildly acidic mycobacteria-containing phagosome (pH 6.1 – 6.5) (Sturgill *et al.*, 1994), which suggests that *sapM* expression is induced within macrophages. To demonstrate that *sapM* is expressed *in vivo*, two transcriptional fusions were constructed. pSAPM-GFP contains 560 bp upstream of the *sapM* coding region and the *sapM* gene truncated 67 bp from its 3' end. pSAPC-GFP contains the same 560 bp *sapM* promoter fragment, the entire *sapM* gene and the *sapC* gene (Rv3311) truncated 183 bp from its 3' end (Fig. 5A). The *sapM* and *sapC* genes appear to be co-transcribed. In both vectors, the truncated genes are transcriptionally fused to a promoterless mutant green fluorescent protein (mGFP) (Barker *et al.*, 1998). These constructs, together with the cloning vector pFPV27, were transformed into *Mycobacterium marinum*, a fish pathogen that also produces SapM from a chromosomal loci (see below). Recombinant *M. marinum* strains grown in asparagine-containing Sauton medium (pH 7.4), in which the *sapM* expression is not induced or at very low levels, were used to infect the murine macrophage-like cell line J774A.1. As predicted, within macrophages, cells of *M. marinum* containing pSAPM-GFP or pSAPC-GFP were brightly fluorescent, whereas the extracellular bacteria had minimal fluorescence (Fig. 5B). *M. marinum* strains containing the cloning vector pFPV27 (Fig. 5B) or the construct in which *sapM* promoter was inserted in the opposite orientation (data not shown) were nonfluorescent. This result, together with our *in vitro* data described above, indicates that *sapM* is induced by the mildly acidic environment of mycobacterial phagosome of cultured macrophages.

The *sapM* gene is present in pathogenic mycobacteria only

As described above, SapM protein and enzyme activity were not detected in *M. smegmatis*, a nonpathogenic *Mycobacterium* species. To examine if SapM is specific to pathogenic mycobacteria, we analyzed two other *Mycobacterium* species, the nonpathogenic *Mycobacterium chelonae* and the fish pathogen, *M. marinum*. Like *M. bovis* BCG, the CFP of *M. marinum* culture grown in ammonium-containing Sauton medium exhibited a high level of acid phosphatase activity (data not shown), and this activity was dependent on the pH of the culture media (data not shown). In contrast, *M. chelonae* was similar to *M. smegmatis* and exhibited a residual level of acid phosphatase activity that was not regulated by media pH (data not shown). These results suggest that *M. marinum* contains a SapM homolog, whereas *M. chelonae* and *M. smegmatis* do not. Searching the unpublished genome sequences available at TIGR and the Sanger center revealed that SapM homologs are present in *M. marinum* and *Mycobacterium avium* (a bird pathogen), but not *M. smegmatis* (Fig. 6A).

To confirm the above results, DNA hybridizations of chromosomal DNA of *M. smegmatis* and *M. chelonae* using a radiolabeled probe specific for *M. tuberculosis sapM* were performed. Chromosomal DNA of *M. bovis* BCG and the *sapM*-containing plasmid pSAP were included as positive controls. The result showed that the *sapM* allele was not detected in *M. smegmatis* and *M. chelonae* chromosomes (Fig. 6B).

Immunogenicity of SapM

The antigenicity of SapM in human infection was assessed by Western blot using partially purified *M. tuberculosis* SapM and sera collected from seven individuals. These individuals included one patient diagnosed with lymph node TB who began chemotherapy four months prior to collection of the serum; one patient who was sputum smear-positive for *M. avium*; two individuals who had been vaccinated with *M. bovis* BCG; three apparently healthy individuals including one involved with patient care and one who tested PPD positive. Interestingly, sera from two patients (TB patient and patient infected by *M. avium*) reacted with SapM (lanes 4 and 5, Fig. 7), whereas sera from two healthy individuals including the one tested for PPD-positive did not react with

SapM (lanes 2 and 6). However, sera from two individuals who had been vaccinated with BCG (lanes 7 and 8) and the individual involved in TB patient care (lane 3) also reacted with SapM. These results suggest that SapM is recognized by antibodies from TB patients, or from individuals who had been vaccinated with BCG, but not from healthy individuals who had not been exposed to *M. tuberculosis*.

Mycobacterium tuberculosis produces a secreted acid phosphatase, SapM, which exhibits remarkably high activity towards GTP and NADPH. The biological functions of SapM are unknown. In this study, we found that *sapM* is regulated by pH. The expression level and enzyme activity of SapM are dramatically increased (up to ~30 fold) when the pH of culture medium is lowered from 7.0 – 7.4 to 5.8 – 6.6. Transcriptional fusions of truncated *sapM* with the promoterless *gfp* demonstrated that *sapM* expression is induced in infected macrophages. DNA hybridizations and sequence analyses indicated that the *sapM* gene is present in pathogenic mycobacteria including *Mycobacterium bovis* BCG, *Mycobacterium avium*, and *Mycobacterium marinum*, but is absent from nonpathogenic mycobacteria such as *Mycobacterium smegmatis* and *Mycobacterium chelonae*. Moreover, antibodies from TB patients or BCG vaccinated individuals but not from healthy individuals recognize SapM. Collectively, these results suggest that SapM is important for mycobacterial pathogenesis, which may contribute to intracellular survival by interfering with effector molecules involved in phagosome maturation.

Materials and Methods

Strains and culture conditions

M. bovis BCG strains, BCG-Japan, -Pasteur, -Frappier, and -Birkhaug, were provided by Marcel Behr (McGill University). *M. marinum* 1218R strain was provided by Lucia Barker (Rocky Mountain Laboratories, NIAID). *M. smegmatis* mc²-155 and *M. chelonae* PS4770 were described previously (Liu *et al.*, 1995; Liu *et al.*, 1996).

Mycobacterial cells were routinely grown in normal Sauton media containing (per liter): 0.5 g KH₂PO₄, 0.14 g MgSO₄, 2.0 g citric acid, 0.05 g ferric ammonium citrate, 5.0 g asparagine, and 60 ml glycerol. The pH was adjusted

to 7.4. To study the effect of phosphate concentration on acid phosphatase activity, the bacteria were grown in Sauton medium to exponential phase, washed with the same medium without KH_2PO_4 and inoculated in 100 ml of Sauton medium in which the phosphate concentration was modified as follows (per liter): KH_2PO_4 was increased by 10 fold to 5.0 g (high Pi medium), or reduced by 10 fold to 0.05 g (low Pi medium). For studying the effect of nitrogen source on acid phosphatase, asparagine (5 g/l) in the original Sauton was substituted with NH_4Cl (1.42 g/l). For carbon source starvation, glycerol was omitted from the Sauton medium. For studying the effect of pH on acid phosphatase activity, the pH of the original Sauton medium was adjusted with buffers: 20 mM MOPS (for pH 7.0 and 6.6), or 20 mM MES (for pH 6.2 and 5.8). All cultures were grown with continuous shaking at 37°C, except for *M. marinum* and *M. chelonae*, which were grown at 30°C.

Molecular cloning

An ordered BAC library of *M. tuberculosis* H37Rv genome was used as DNA template for cloning. Standard protocols were used for manipulation of DNA. Cloning of *sapM* was accomplished by ligating a 3 kb *NheI* fragment of BAC403, containing Rv3309 (*upp*), Rv3310 (*sapM*) and Rv3311 (*sapC*), into the unique *XbaI* site of plasmid pMD31 to generate pSAP. Transcriptional fusions to a promoterless mutant *gfp* were constructed by cloning fragments of pSAP into pFPV27. To construct pSAPC-GFP, a 2.56 kb *EcoRV* fragment of pSAPM, comprising 560 bp upstream of *sapM*, the complete *sapM* gene, and *sapC* (Rv3311) truncated 183 bp from its 3' end, was inserted into the unique *EcoRV* site of pFPV27 (Barker *et al.*, 1998). To generate pSAPM-GFP, vector pSAPC-GFP was cut with *BglII* and the 3.8 kb and 2.5 kb fragments, comprising pFPV27, 560 bp upstream of the *sapM* start codon and all but 67 bp of the *sapM* coding region, were re-ligated. Plasmids were introduced into *M. marinum* and *M. smegmatis* mc²-155 by electroporation, and recombinants were selected on Middlebrook 7H9 agar (Difco) supplemented with 10% OADC enrichment and 25 µg/ml kanamycin.

Southern blotting

Southern blotting was performed according to standard methods. Briefly, chromosomal DNA was isolated from *M. bovis* BCG, *M. smegmatis* mc²-155, and *M. chelonae*, digested with *EcoRI* or *EcoRV*, separated on an agarose gel and transferred to a nylon membrane (Hybond N+, Amersham Pharmacia). Membranes were then hybridized with $\alpha^{32}\text{P}$ -CTP radiolabeled *sapM* probe (a 660 bp *HphI*-*NdeI* fragment of pSAP) and exposed on a phosphorimager cassette.

Enzyme assays

For assay of secreted acid phosphatase activity, the culture filtrate protein (CFP) fractions were collected from cultures grown to exponential phase ($A_{600} \sim 0.8$), concentrated by centrifugal filters (Millipore), and dialyzed (150-200 \times volumes) against distilled water overnight at 4°C. Protein concentrations were determined using the BCA assay (Pierce). Acid phosphatase assay was carried out using *p*-nitrophenylphosphate (pNPP) at pH 6.8 in a microtitre format as described previously (Saleh and Belisle, 2000). Specific activity was calculated using an extinction coefficient for nitrophenol of 18,380 $\text{mM hr}^{-1} \text{cm}^{-1}$, and the phosphatase activity was expressed as $\text{nmol hr}^{-1} \text{mg}^{-1}$ (total CFP protein).

For assay of cell surface-associated acid phosphatase and alkaline phosphatase, bacterial cells were collected and briefly sonicated to disrupt cell clumps. Cells were then washed in Tris-buffered saline three times followed by a final wash in distilled water and cell density was adjusted to a final OD_{595} of 0.42. The phosphatase activity of whole cells toward pNPP was assayed by measuring *p*-nitrophenol released as described above. Typically, 50 μl of bacterial cells were mixed with 150 μl of 25 mM acetate buffer, pH 6.0 (for acid phosphatase assay) or 25 mM Tris, pH 10.0 (for alkaline phosphatase). Reaction buffers also contained 1 mM each of MgCl_2 and ZnCl_2 . The reactions were initiated by the addition of pNPP to a final concentration of 1 mg/ml. Following incubation at 37°C for 30 min., cells were pelleted by centrifugation and the top 150 μl of the reactions were transferred

to ELISA plate wells containing 50 μ l of 1.0 M NaOH, which stopped the reaction, and the OD_{405nm} measured in an ELISA reader.

Generation of anti-SapM antisera

A peptide, corresponding to residues 201-208 in the SapM amino acid sequence, was synthesized using solid phase peptide synthesis. The peptide was modified by acetylation of the carboxyl end and by the addition of a cysteine at the amino end. Approximately 10 mg of the purified peptide, having the sequence CNDGHDGSI-Ac, was reduced by dissolving in 4.0 ml of 10 mM ammonium bicarbonate buffer, pH 8.0 containing 20 mM DTT (buffer A) and incubating at room temperature for 2 hours. The reduced peptide was loaded onto an 1.5 cm \times 4.0 cm anion-exchange column (DEAE-Sephadex A50, Pharmacia) pre-equilibrated in buffer A, followed by washing with 25 ml of same buffer without DTT. Bound peptide was eluted with 10 ml of 10 mM acetate buffer, pH 3.0. The peptide, in a total volume of 2.0 ml, was immediately added to a tube containing 2 mg of mCKLH (Pierce Sciences), followed by the addition of 200 μ l of 1.0 M phosphate buffer, pH 7.4 and mixed continuously at room temperature for 2 hours. The conjugated KLH was subsequently desalted on a G-10 column (1.5 cm \times 25 cm) in PBS. Two New Zealand rabbits were primed with 250 μ g of the KLH conjugate, followed by a first boost at 28 days and a second boost at 56 days (100 μ g each). Sera were screened 9 days following the second boost and the antisera collected the following day.

SDS-polyacrylamide gel electrophoresis and Western blotting

For detection of SapM protein, CFP samples (typically 8-10 μ g protein) were separated on sodium dodecyl sulfate (SDS)-14% polyacrylamide gels, and visualized by silver staining, or for Western blotting, transferred to nitrocellulose membranes. The membranes were then probed with a 1:400 dilution of anti-SapM antisera and developed with anti-rabbit IgG-alkaline phosphatase conjugate and BCIP/NBT. For assay of SapM antigenicity, SapM protein was partially purified from the recombinant *M. smegmatis* strain carrying the plasmid pSAP, using methods described previously (Saleh and

Belisle, 2000). The protein was run on 14% SDS-PAGE and transferred to nitrocellulose membranes. Individual lanes were cut and probed with a 1:150 dilution of individual human sera and developed as described above. Human serum samples were provided by Tony Mazzulli (Mount Sinai Hospital, Toronto, ON).

Fluorescence microscopy

The mouse macrophage cell line J774 was maintained in RPMI 1640 media containing fetal bovine serum (5 %), 100 μ g / ml streptomycin, and 100 units / ml penicillin. For infections with *M. marinum*, cells were allowed to adhere to glass cover slips (22 \times 22 mm) overnight in incubator at 37°C, 5 % CO₂. Spent media was then replaced with fresh media (without antibiotics) and *M. marinum* was added at MOI of 10:1 and incubated at 37°C for 1 hr. Excess bacilli were removed by several washes with sterile PBS and the infected cells were finally covered with fresh media and returned to the incubator. At given times post infection, the cover slips were washed several times with PBS, fixed for 15 minutes with 3.7% paraformaldehyde, permeabilized for 10 minutes with 0.1 % Triton X-100, and stained with Texas-red phalloidin for 1 hr. All treatments were at room temperature. Images were collected on a Leica DM IRBE microscope equipped with a Hamamatsu digital camera. Images were collected using differential interference contrast (DIC), with a FITC filter to visualize GFP, or with a Texas red filter to visualize macrophages treated with cytoskeleton-specific stain phalloidin conjugated with Texas red.

The present invention has been described in detail and with particular reference to the preferred embodiments; however, it will be understood by one having ordinary skill in the art that changes can be made without departing from the spirit and scope thereof. For example, where the application refers to proteins, it is clear that peptides and polypeptides may often be used. Likewise, where a gene is described in the application, it is clear that nucleic acids or gene fragments may often be used.

All publications (including Genbank entries), patents and patent applications are incorporated by reference in their entirety to the same extent as if each

individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

References

- Barker, L.P., Brooks, D.M., and Small, P.L. (1998) The identification of *Mycobacterium marinum* genes differentially expressed in macrophage phagosomes using promoter fusions to green fluorescent protein. *Mol Microbiol* **29**: 1167-1177.
- Berthet, F.X., Lagranderie, M., Gounon, P., Laurent, W., Ensergueix, D., Chavarot, P., Thouron, F., Maranghi, E., Pelicic, V., Portnoi, D., Marchal, G., and Gicquel, B. (1998) Attenuation of virulence by disruption of the *Mycobacterium tuberculosis* *erp* gene. *Science* **282**: 759-762.
- Betts, J.C., Lukey, P.T., Robb, L.C., McAdam, R.A., and Duncan, K. (2002) Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. *Mol Microbiol* **43**: 717-731.
- Braibant, M., and Content, J. (2001) The cell surface associated phosphatase activity of *Mycobacterium bovis* BCG is not regulated by environmental inorganic phosphate. *FEMS Microbiol Lett* **195**: 121-126.
- Clemens, D.L., and Horwitz, M.A. (1995) Characterization of the *Mycobacterium tuberculosis* phagosome and evidence that phagosomal maturation is inhibited. *J Exp Med* **181**: 257-270.
- Clemens, D.L., Lee, B.Y., and Horwitz, M.A. (2000) Deviant expression of Rab5 on phagosomes containing the intracellular pathogens *Mycobacterium tuberculosis* and *Legionella pneumophila* is associated with altered phagosomal fate. *Infect Immun* **68**: 2671-2684.
- Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S.V., Eiglmeier, K., Gas, S., Barry, C.E., Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., and Barrell, B.G. (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**: 537-544.
- Dowling, J.N., Saha, A.K., and Glew, R.H. (1992) Virulence factors of the family *Legionellaceae*. *Microbiol Rev* **56**: 32-60.
- Fisher, M.A., Plikaytis, B.B., and Shinnick, T.M. (2002) Microarray analysis of the *Mycobacterium tuberculosis* transcriptional response to the acidic conditions found in phagosomes. *J Bacteriol* **184**: 4025-4032.

- Kuehnel, M.P., Goethe, R., Habermann, A., Mueller, E., Rohde, M., Griffiths, G., and Valentin, W. (2001) Characterization of the intracellular survival of *Mycobacterium avium* ssp. paratuberculosis: phagosomal pH and fusogenicity in J774 macrophages compared with other mycobacteria. *Cell Microbiol* **3**: 551-566.
- Liu, J., Rosenberg, E.Y., and Nikaido, H. (1995) Fluidity of the lipid domain of cell wall from *Mycobacterium chelonae*. *Proc Natl Acad Sci U S A* **92**: 11254-11258.
- Liu, J., Takiff, H.E., and Nikaido, H. (1996) Active efflux of fluoroquinolones in *Mycobacterium smegmatis* mediated by LfrA, a multidrug efflux pump. *J Bacteriol* **178**: 3791-3795.
- Lucas, R.L., and Lee, C.A. (2000) Unraveling the mysteries of virulence gene regulation in *Salmonella typhimurium*. *Mol Microbiol* **36**: 1024-1033.
- McKinney, J.D., Honer, Z., Munoz, E., Miczak, A., Chen, B., Chan, W.T., Swenson, D., Sacchettini, J.C., Jacobs, W.R., and Russell, D.G. (2000) Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature* **406**: 735-738.
- Reilly, T.J., Baron, G.S., Nano, F.E., and Kuhlenschmidt, M.S. (1996) Characterization and sequencing of a respiratory burst-inhibiting acid phosphatase from *Francisella tularensis*. *J Biol Chem* **271**: 10973-10983.
- Rossolini, G.M., Schippa, S., Riccio, M.L., Berlutti, F., Macaskie, L.E., and Thaller, M.C. (1998) Bacterial nonspecific acid phosphohydrolases: physiology, evolution and use as tools in microbial biotechnology. *Cell Mol Life Sci* **54**: 833-850.
- Saleh, M.T., and Belisle, J.T. (2000) Secretion of an acid phosphatase (SapM) by *Mycobacterium tuberculosis* that is similar to eukaryotic acid phosphatases. *J Bacteriol* **182**: 6850-6853.
- Sturgill, K., Schlesinger, P.H., Chakraborty, P., Haddix, P.L., Collins, H.L., Fok, A.K., Allen, R.D., Gluck, S.L., Heuser, J., and Russell, D.G. (1994) Lack of acidification in *Mycobacterium* phagosomes produced by exclusion of the vesicular proton-ATPase. *Science* **263**: 678-681.
- Takai, Y., Sasaki, T., and Matozaki, T. (2001) Small GTP-binding proteins. *Physiol Rev* **81**: 153-208.

Teitelbaum, R., Cammer, M., Maitland, M.L., Freitag, N.E., Condeelis, J., and Bloom, B.R. (1999) Mycobacterial infection of macrophages results in membrane-permeable phagosomes. *Proc Natl Acad Sci U S A* **96**: 15190-15195.

Triccas, J.A., and Gicquel, B. (2000) Life on the inside: probing *Mycobacterium tuberculosis* gene expression during infection. *Immunol Cell Biol* **78**: 311-317.

Via, L.E., Deretic, D., Ulmer, R.J., Hibler, N.S., Huber, L.A., and Deretic, V. (1997) Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7. *J Biol Chem* **272**: 13326-13331.

Westhoff, C.M., Ferreri, J., Mak, D.O., and Foskett, J.K. (2002) Identification of the erythrocyte Rh blood group glycoprotein as a mammalian ammonium transporter. *J Biol Chem* **277**: 12499-12502.

Xu, S., Cooper, A., Sturgill, K., van, H., Chatterjee, D., Orme, I., Allen, P., and Russell, D.G. (1994) Intracellular trafficking in *Mycobacterium tuberculosis* and *Mycobacterium avium*-infected macrophages. *J Immunol* **153**: 2568-2578.

Yuan, Y., Crane, D.D., Simpson, R.M., Zhu, Y.Q., Hickey, M.J., Sherman, D.R., and Barry, C.E. (1998) The 16-kDa alpha-crystallin (Acr) protein of *Mycobacterium tuberculosis* is required for growth in macrophages. *Proc Natl Acad Sci U S A* **95**: 9578-9583.

We claim:

1. An isolated DNA sequence comprising a promoter or a promoter fragment of a mycobacterial secreted acid phosphatase gene wherein the promoter or the promoter fragment is sufficient to control expression of a nucleotide sequence of interest and is inducible under low-pH conditions.
2. The isolated DNA sequence of claim 1, wherein the promoter or the promoter fragment is selected from the group consisting of a *Mycobacterium tuberculosis* sapM promoter or promoter fragment, a *Mycobacterium bovis* sapM promoter or promoter fragment, a *Mycobacterium avium* sapM promoter or promoter fragment, and a *Mycobacterium marinum* sapM promoter or promoter fragment.
3. An isolated DNA sequence comprising a promoter or a promoter fragment sufficient to control expression of a nucleotide sequence of interest and inducible under low-pH conditions, wherein the promoter or the promoter fragment hybridize to a sapM promoter selected from the group consisting of a *Mycobacterium tuberculosis* sapM promoter [SEQ ID NO:1], a *Mycobacterium bovis* sapM promoter [SEQ ID NO:2], a *Mycobacterium avium* sapM promoter [SEQ ID NO:3] and a *Mycobacterium marinum* sapM promoter [SEQ ID NO:4] under high stringency hybridization conditions.
4. An expression vector comprising the isolated DNA sequence of claim 1, 2 or 3.
5. A host cell transformed with the vector of claim 4.
6. A transcription cassette comprising:
 - (a) a mycobacterial secreted acid phosphatase promoter or promoter fragment, wherein the promoter or the promoter fragment is sufficient to control expression of a nucleotide sequence of interest;
 - (b) a nucleotide sequence of interest operably linked to the promoter or the promoter fragment; and

(c) a transcriptional termination region.

7. The transcription cassette of claim 6 wherein the mycobacterial secreted acid phosphatase promoter or promoter fragment is selected from the group consisting of a *Mycobacterium tuberculosis* sapM promoter [SEQ ID NO:1], a *Mycobacterium bovis* sapM promoter [SEQ ID NO:2], a *Mycobacterium avium* sapM promoter [SEQ ID NO:3], and a *Mycobacterium marinum* sapM promoter [SEQ ID NO:4].

8. The transcription cassette of claim 6 or 7 further comprising a mycobacterial secreted acid phosphatase N-terminal signal sequence.

9. The transcription cassette of claim 8 wherein the mycobacterial secreted acid phosphatase N-terminal signal sequence is selected from the group consisting of a *Mycobacterium tuberculosis* sapM N-terminal signal sequence [SEQ ID NO:5], a *Mycobacterium bovis* sapM N-terminal signal sequence [SEQ ID NO:6], a *Mycobacterium avium* sapM N-terminal signal sequence [SEQ ID NO:7], and a *Mycobacterium marinum* sapM N-terminal signal sequence [SEQ ID NO:8].

10. A method for the diagnosis of a pathogenic mycobacterial infection or a pathogenic fungal infection in a subject comprising:

- (a) obtaining a biological sample from a subject; and
- (b) analyzing the sample for the presence of antibodies specific to a mycobacterial secreted acid phosphatase wherein detection of antibodies specific to the mycobacterial secreted acid phosphatase is indicative of the presence of the pathogenic mycobacterial infection or the pathogenic fungal infection.

11. The method of claim 10, wherein the mycobacterial secreted acid phosphatase is selected from the group consisting of a *Mycobacterium tuberculosis* SapM [SEQ ID NO:10], a *Mycobacterium bovis* SapM [SEQ ID NO:12], a *Mycobacterium avium* SapM [SEQ ID NO:14], and a *Mycobacterium marinum* SapM [SEQ ID NO:16].

12. A method for the diagnosis of a pathogenic mycobacterial infection or a pathogenic fungal infection in a subject comprising:

- (a) obtaining a nucleic acid sample from a subject; and
- (b) analyzing the sample for the presence of a nucleic acid encoding a mycobacterial secreted acid phosphatase wherein detection of the nucleic acid encoding the mycobacterial secreted acid phosphatase is indicative of the presence of the pathogenic mycobacterial infection or a pathogenic fungal infection.

13. The method of claim 12 wherein the nucleic acid encoding the mycobacterial secreted acid phosphatase is selected from the group consisting of a *Mycobacterium tuberculosis* sapM, a *Mycobacterium bovis* sapM, a *Mycobacterium avium* sapM, and a *Mycobacterium marinum* sapM.

14. A method for the diagnosis of a pathogenic mycobacterial infection or a pathogenic fungal infection in a subject comprising:

- (a) obtaining a biological sample from a subject; and
- (b) analyzing the sample for the presence of a mycobacterial secreted acid phosphatase activity, wherein detection of the mycobacterial secreted acid phosphatase activity is indicative of the presence of the pathogenic mycobacterial infection or the pathogenic fungal infection.

15. The method of claim 14, wherein the mycobacterial secreted acid phosphatase activity is selected from the group of mycobacteria consisting of a *Mycobacterium tuberculosis* SapM activity, a *Mycobacterium bovis* SapM activity, a *Mycobacterium avium* SapM activity, and a *Mycobacterium marinum* SapM activity.

16. A method of screening for a compound capable of modulating production of a mycobacterial secreted acid phosphatase comprising:

- (a) providing a nucleic acid construct comprising a mycobacterial secreted acid phosphatase promoter or promoter fragment, wherein the promoter or

the promoter fragment is operably linked to a reporter gene capable of producing a measurable signal;

- (b) providing a test compound;
- (c) exposing the nucleic acid construct to the test compound; and
- (d) measuring the signal produced by the reporter gene, wherein a change in the signal produced in the presence of the test compound as compared to in the absence of the test compound indicates that the test compound is capable of modulating production of the mycobacterial secreted acid phosphatase.

17. The method of claim 16, wherein the mycobacterial secreted acid phosphatase promoter or promoter fragment is selected from the group consisting of a *Mycobacterium tuberculosis* sapM promoter or promoter fragment, a *Mycobacterium bovis* sapM promoter or promoter fragment, a *Mycobacterium avium* sapM promoter or promoter fragment, and a *Mycobacterium marinum* sapM promoter or promoter fragment.

18. A method of screening for a compound capable of modulating the activity of a mycobacterial secreted acid phosphatase comprising:

- (a) incubating a mixture comprising a mycobacterial secreted acid phosphatase, a substrate for the mycobacterial secreted acid phosphatase, and a test compound; and
- (b) measuring the activity of the mycobacterial secreted acid phosphatase; wherein a change in activity in the presence of the test compound as compared to in the absence of the test compound indicates that the test compound is capable of modulating the activity of the secreted acid phosphatase.

19. The method of claim 18, wherein the mycobacterial secreted acid phosphatase is selected from the group consisting of a *Mycobacterium tuberculosis* SapM, a *Mycobacterium bovis* SapM, a *Mycobacterium avium* SapM, and a *Mycobacterium marinum*.

20. A method of screening for a compound capable of modulating secretion of a mycobacterial secreted acid phosphatase comprising:

- (a) exposing mycobacterium cells to a test compound, wherein the mycobacterium cells secrete a mycobacterial secreted acid phosphatase and
- (b) measuring the amount of the mycobacterial secreted acid phosphatase, secreted by the mycobacterium cells, wherein a change in the secretion of the mycobacterial secreted acid phosphatase in the presence of the test compound as compared to in the absence of the test compound indicates that the test compound is capable of modulating the secretion of the mycobacterial secreted phosphatase.

21. The method of claim 20, wherein the mycobacterial secreted acid phosphatase is selected from the group consisting of a *Mycobacterium tuberculosis* SapM, a *Mycobacterium bovis* SapM, a *Mycobacterium avium* SapM, and a *Mycobacterium marinum* SapM.

22. A kit for the detection of a pathogenic mycobacterial disease or infection comprising:

- (a) a mycobacterial secreted acid phosphatase;
- (b) at least one antibody specific for the mycobacterial secreted acid phosphatase ; and
- (c) one or more reagents necessary for detection of the antibody.

23. The kit of claim 22, wherein the mycobacterial secreted acid phosphatase is selected from the group consisting of *Mycobacterium tuberculosis* SapM, *Mycobacterium bovis* SapM, *Mycobacterium avium* SapM, and *Mycobacterium marinum* SapM.

24. A kit for the detection of a pathogenic mycobacterial disease or infection comprising:

- (a) an oligonucleotide comprising contiguous nucleotides from the nucleic acid sequence that is complementary to the sequence of [SEQ ID NO:1] to [SEQ ID NO:4], or [SEQ ID NO:9], [SEQ ID NO:11], [SEQ ID NO:13], [SEQ ID

NO:15] and capable of specifically hybridizing to the complementary nucleotide sequence; and

(b) reagents for hybridization of the oligonucleotide to a complementary nucleic acid sequence.

25. An antibody which is capable of specifically binding to a mycobacterial secreted acid phosphatase or a polypeptide fragment thereof.

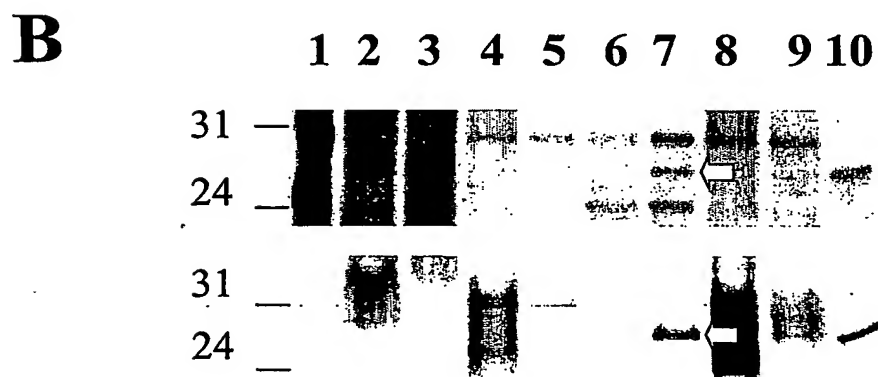
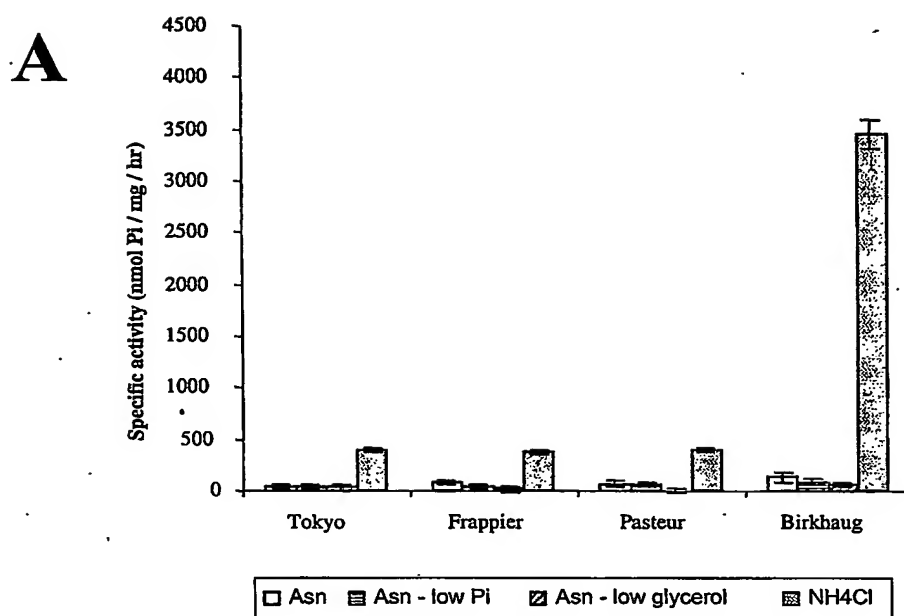
26. The antibody of claim 25, wherein the mycobacterial secreted acid phosphatase is selected from the group consisting of *Mycobacterium tuberculosis* SapM [SEQ ID NO:10], *Mycobacterium bovis* SapM [SEQ ID NO:12], *Mycobacterium avium* SapM [SEQ ID NO:14], and *Mycobacterium marinum* [SEQ ID NO:16] SapM.

27. A vaccine or immunogenic composition for treatment or prophylaxis of a mammal against challenge by a mycobacterium comprising the antibody of claim 26.

28. A vaccine or immunogenic composition for treatment or prophylaxis of a mammal against challenge by a mycobacterium comprising the isolated DNA sequence claim 1,2 or 3.

29. A vaccine or immunogenic composition for the treatment or prophylaxis of a mammal against challenge by a mycobacterium comprising a mycobacterial secreted acid phosphatase selected from the group consisting of a *Mycobacterium tuberculosis* SapM, a *Mycobacterium bovis* SapM, a *Mycobacterium avium* SapM, and a *Mycobacterium marinum* SapM or a polypeptide fragment thereof.

30. An antigenic composition useful for the detection of a pathogenic mycobacterial disease or infection in a subject comprising a SapM polypeptide and substantially free from other proteins or glycoproteins with which it is natively admixed in a culture of pathogenic mycobacteria.

*Figure 1*

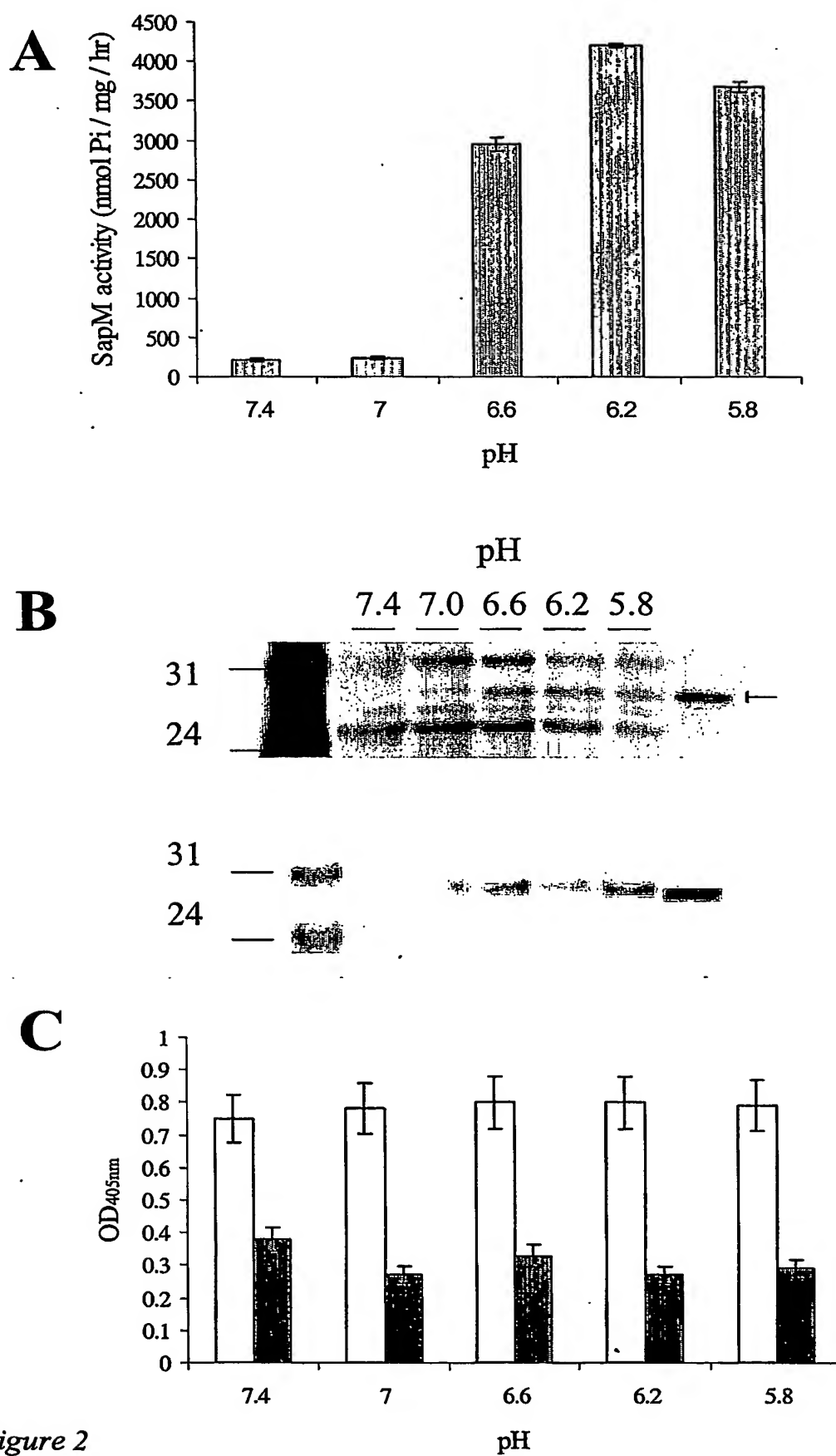
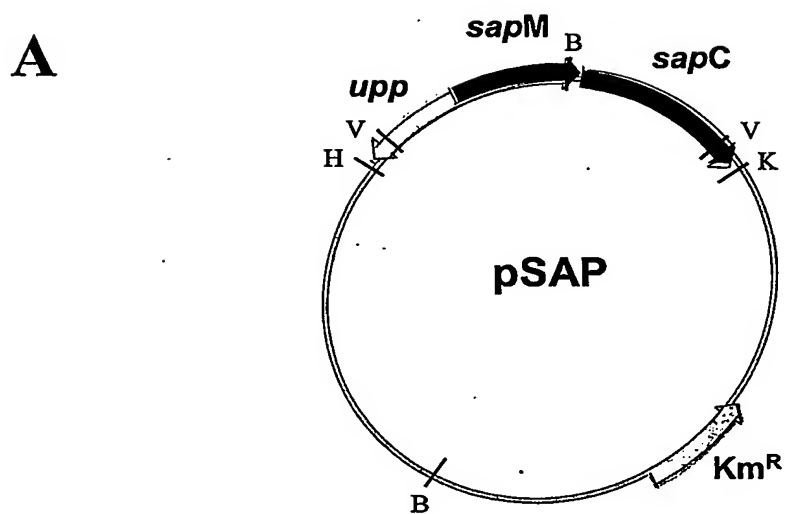


Figure 2



B

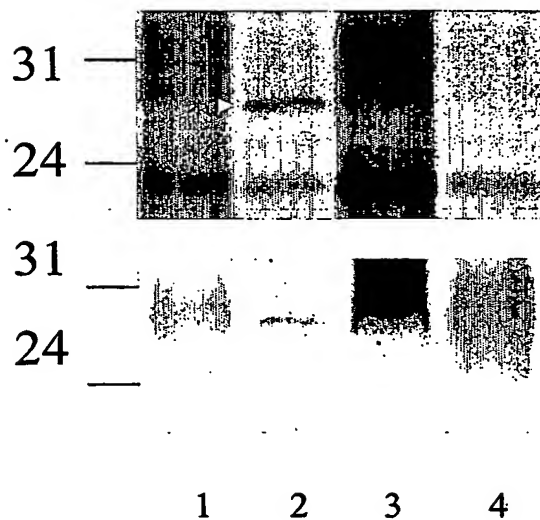
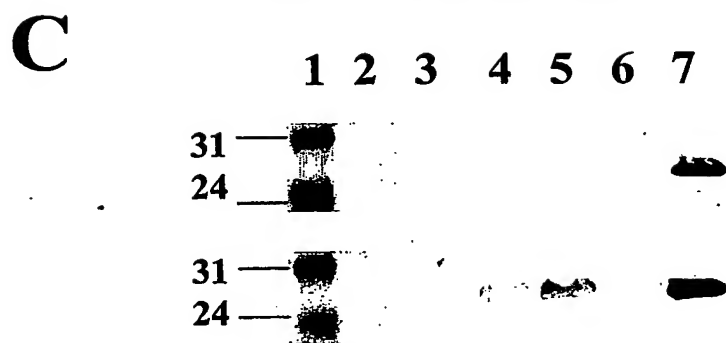
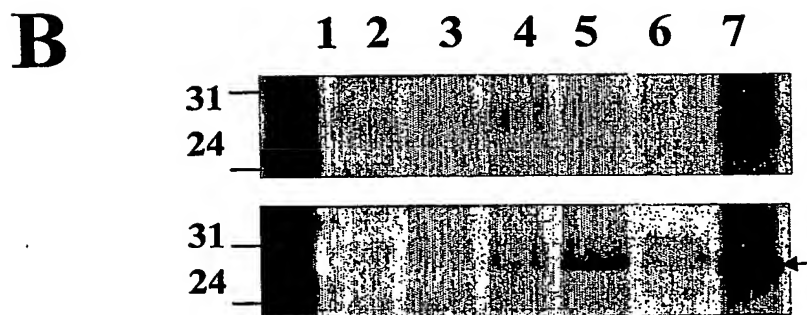
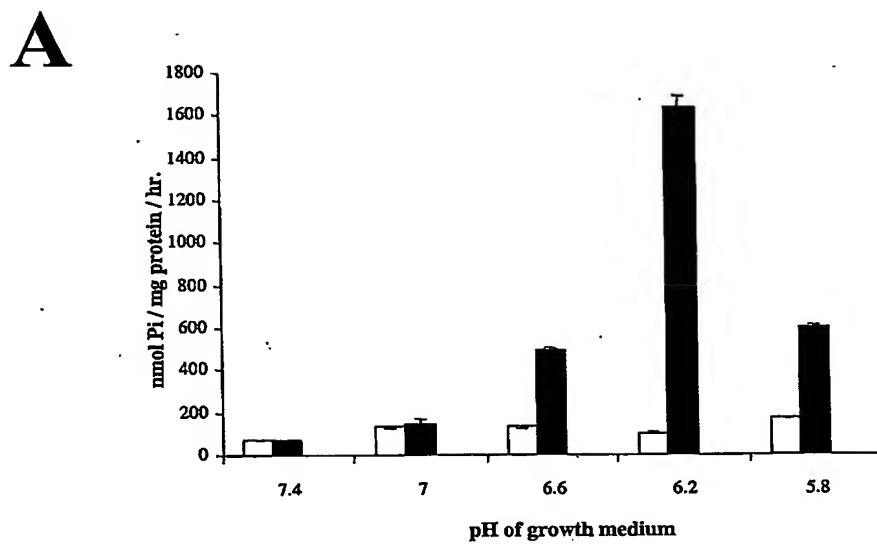


Figure 3

*Figure 4*

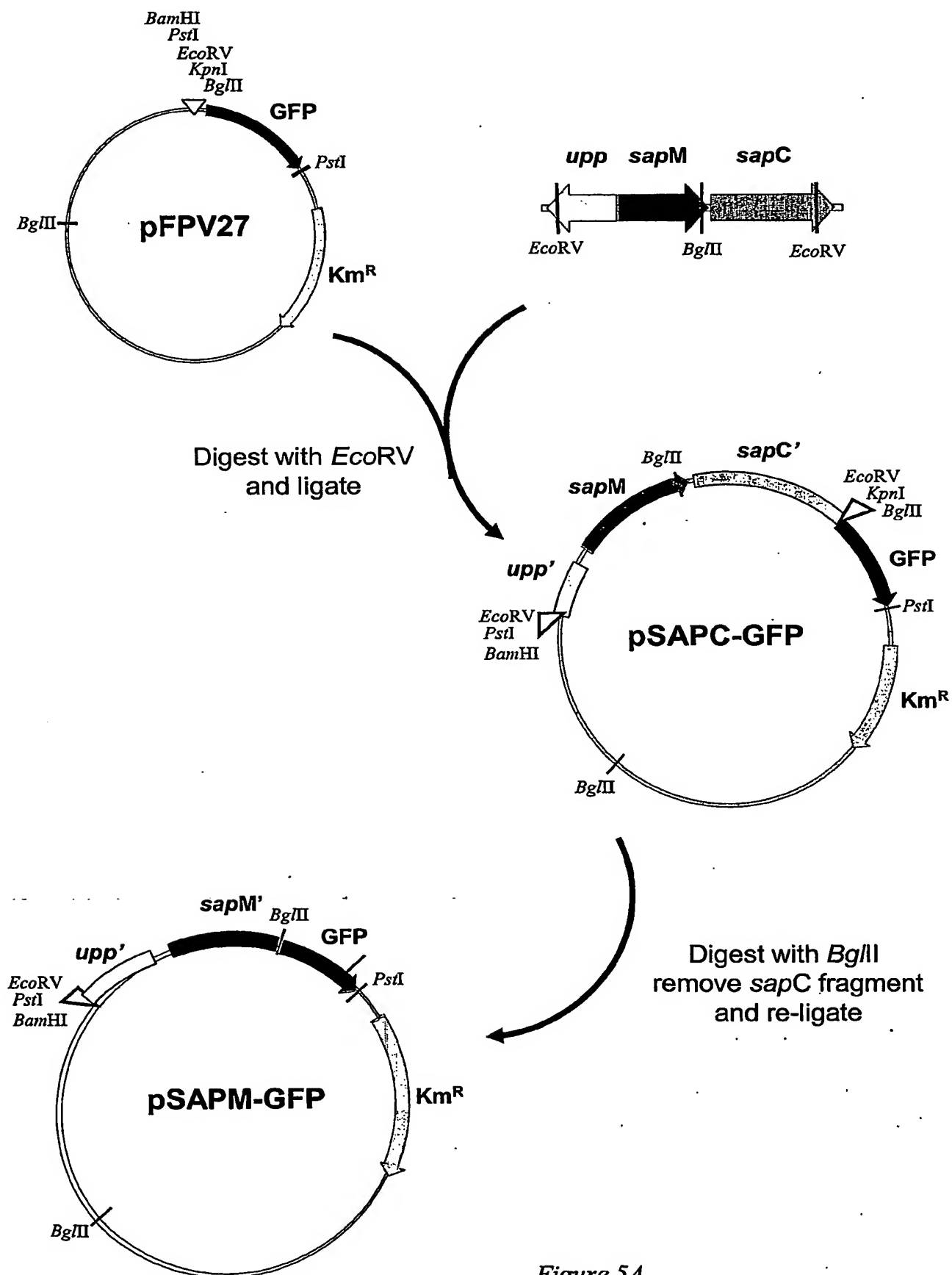


Figure 5A

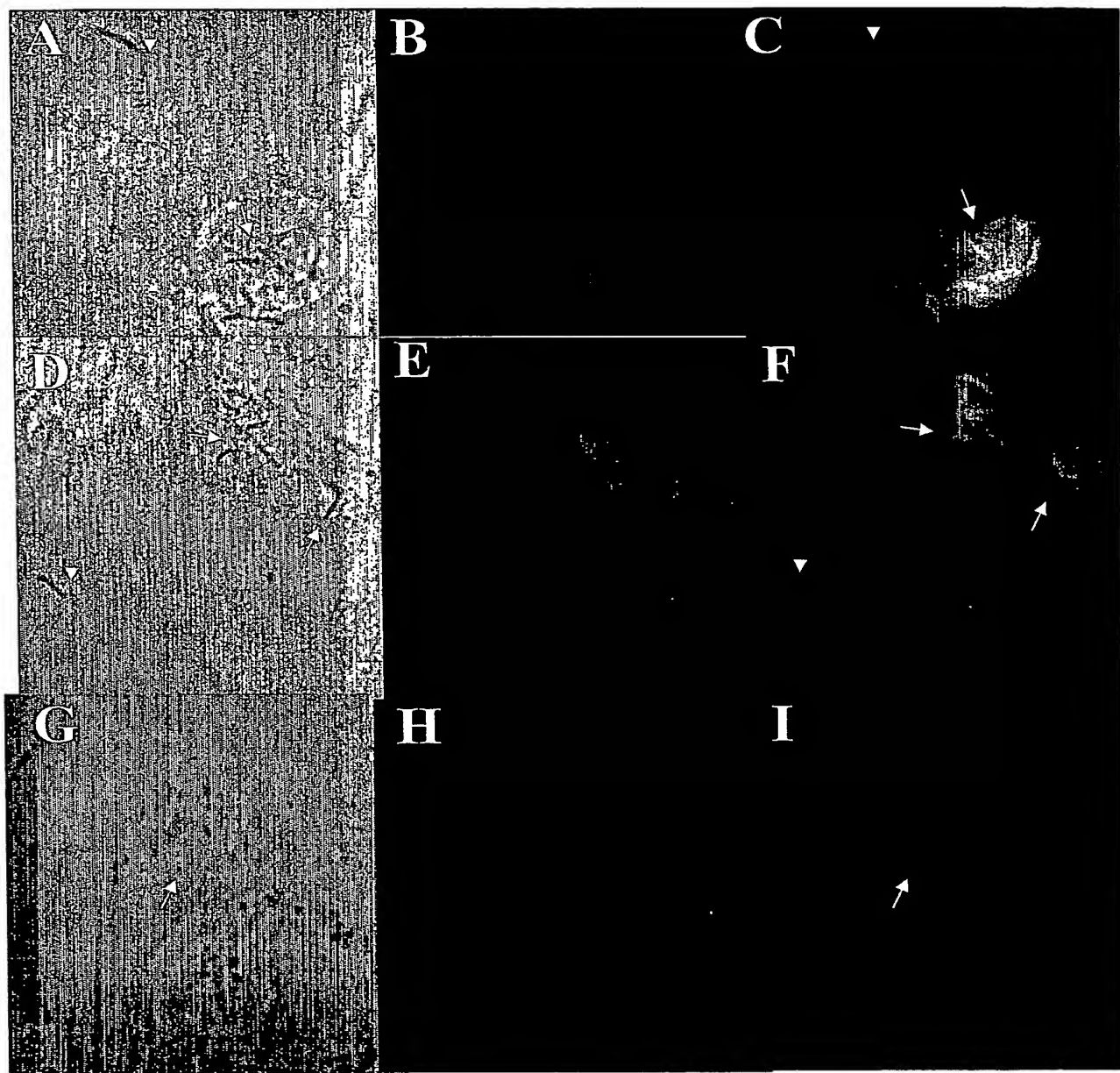
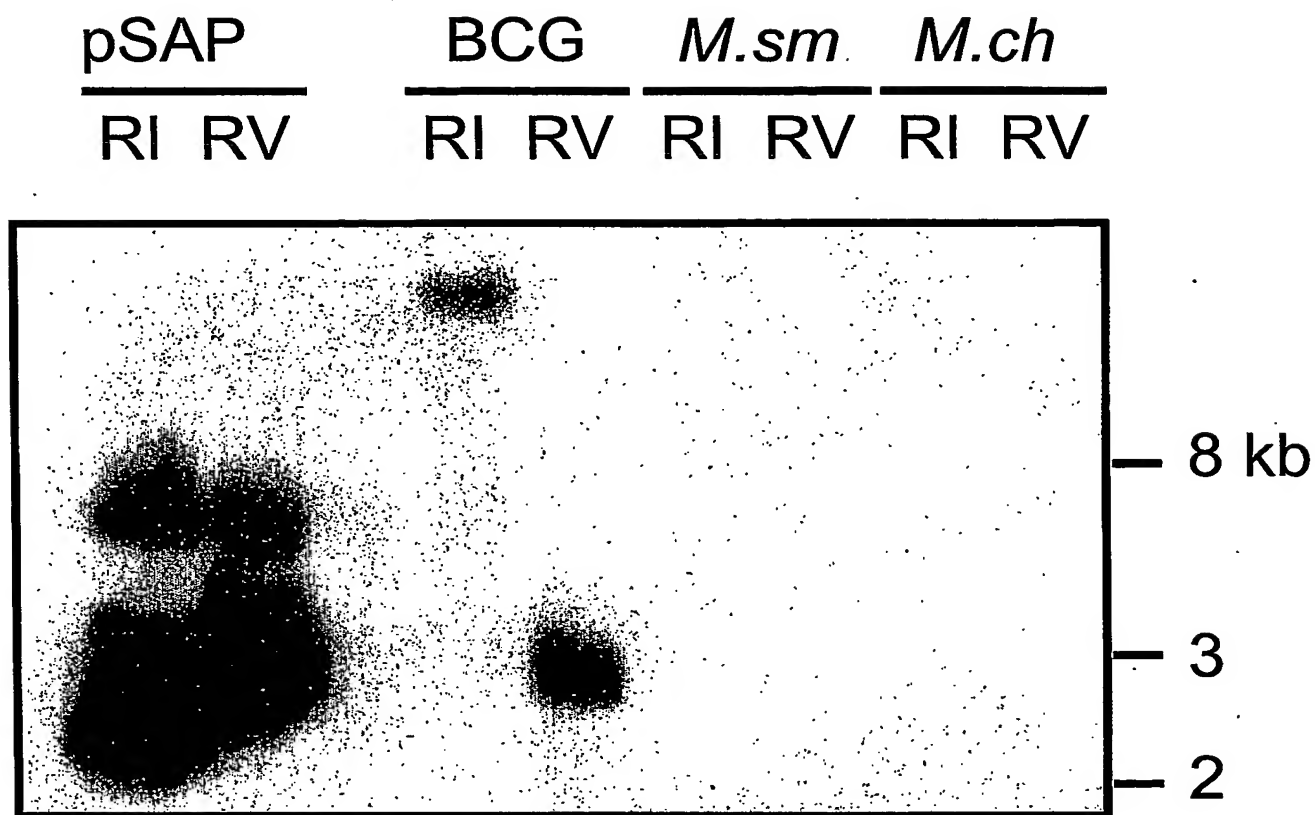


Figure 5B

A

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<i>M.bv</i>	M.....			
<i>M.mr</i>	MCG.KQRF.STF.....L.AV.V..P.HGSD.P.RID.T.T...A.S...V..R..			
<i>M.av</i>	MS.ENRSRR.LIGGA..S.RLL.AV..VA..A-SPLT.RTS...A.I.QPS.I.....			
	61	80	100	120
	+-----+-----+-----+-----+			
<i>M.tb</i>	RSQAAIIGNKSAPFINS LAANGAMMAQAFETHPSEPNYLALFAGNTFGLTKNTCPVNGG			
<i>M.bv</i>			
<i>M.mr</i>	H...N....A....A.....S.S.....T....K.....A.			
<i>M.av</i>	..ESG.....TA...S..N.T.S.....V..DL....A.			
	121	140	160	180
	+-----+-----+-----+-----+			
<i>M.tb</i>	ALPNLGSELLSAGYTFMGFAEDLPAVGSTVCSAGKYARKHVPWVNFSNVPTTLLSVPFSAF			
<i>M.bv</i>A.....			
<i>M.mr</i>	ST...A....A..H..V....S..E.....G...A.....A...M.....			
<i>M.av</i>	.A.....A.....A.Y..G..SP..P.....A..T...AAS.L.....			
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	+-----+-----+-----+-----+			
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<i>M.bv</i>			
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<i>M.av</i>	.M-G..AS.....N..N.....A.....Q..G.....LA.....I..F...			
	241	260	280	300
	+-----+-----+-----+-----+			
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<i>M.bv</i>			
<i>M.mr</i>	----G.....Q.....M.....F.A...V.A....G			
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Fig. 6

B*Fig. 6*

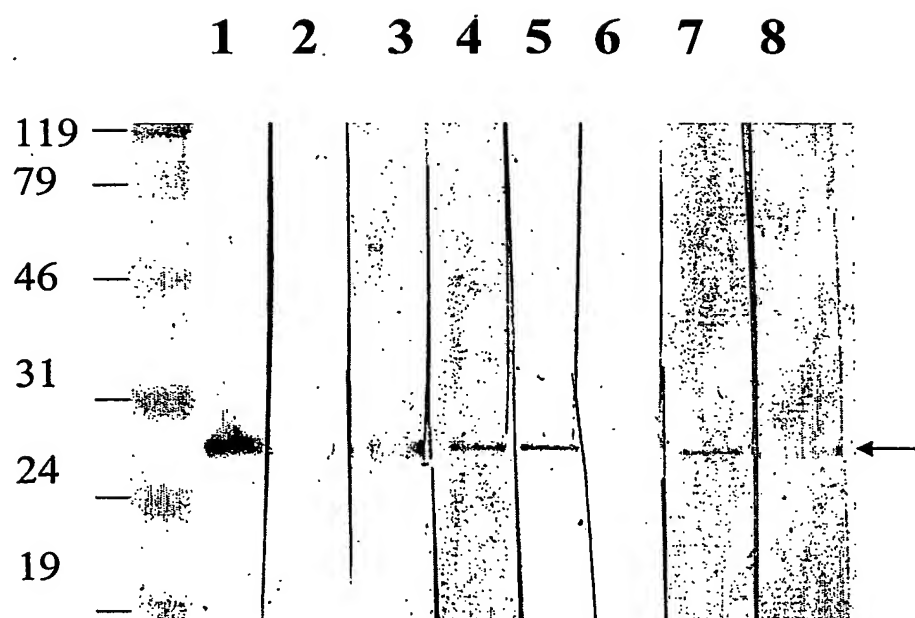


Figure 7

SEQUENCE LISTING

<110> Saleh, Mazen T.

Alexander, David C.

Liu, Jun

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Trp Val Gly Ala Val Pro Gln Val Gly Leu Ala Ala Ser Ala Leu Pro
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Thr Phe Ala His Val Val Ile Val Val Glu Glu Asn Arg Ser Gln Ala
 50 55 60

Ala Ile Ile Gly Asn Lys Ser Ala Pro Phe Ile Asn Ser Leu Ala Ala
 65 70 75 80

Asn Gly Ala Met Met Ala Gln Ala Phe Ala Glu Thr His Pro Ser Glu
 85 90 95

Pro Asn Tyr Leu Ala Leu Phe Ala Gly Asn Thr Phe Gly Leu Thr Lys
 100 105 110

Asn Thr Cys Pro Val Asn Gly Gly Ala Leu Pro Asn Leu Gly Ser Glu
 115 120 125

Leu Leu Ser Ala Gly Tyr Thr Phe Met Gly Phe Ala Glu Asp Leu Pro
 130 135 140

Ala Val Gly Ser Thr Val Cys Ser Ala Gly Lys Tyr Ala Arg Lys His
 145 150 155 160

Val Pro Trp Val Asn Phe Ser Asn Val Pro Thr Thr Leu Ser Val Pro
 165 170 175

Phe Ser Ala Phe Pro Lys Pro Gln Asn Tyr Pro Gly Leu Pro Thr Val
 180 185 190

Ser Phe Val Ile Pro Asn Ala Asp Asn Asp Met His Asp Gly Ser Ile
 195 200 205

Ala Gln Gly Asp Ala Trp Leu Asn Arg His Leu Ser Ala Tyr Ala Asn
 210 215 220

Trp Ala Lys Thr Asn Asn Ser Leu Leu Val Val Thr Trp Asp Glu Asp
 225 230 235 240

Asp Gly Ser Ser Arg Asn Gln Ile Pro Thr Val Phe Tyr Gly Ala His
245 250 255

Val Arg Pro Gly Thr Tyr Asn Glu Thr Ile Ser His Tyr Asn Val Leu
260 265 270

Ser Thr Leu Glu Gln Ile Tyr Gly Leu Pro Lys Thr Gly Tyr Ala Thr
275 280 285

Asn Ala Pro Pro Ile Thr Asp Ile Trp Gly Asp
290 295

<210> 11

<211> 900

<212> DNA

<213> Mycobacterium bovis

<220>

<221> CDS

<222> (1)..(900)

<400> 11

atg ctc cgc gga atc cag gct ctc agc cgg ccc ctg acc agg gta tac 48
Met Leu Arg Gly Ile Gln Ala Leu Ser Arg Pro Leu Thr Arg Val Tyr
1 5 10 15

cgt gcc ttg gcg gtg atc ggt gtc ctg gca gca tcg ttg ctg gcc tca 96
Arg Ala Leu Ala Val Ile Gly Val Leu Ala Ala Ser Leu Leu Ala Ser
20 25 30

tgg gtc ggc gct gtc cca caa gtg ggt ctg gca gcg agt gcc ctg ccg 144
Trp Val Gly Ala Val Pro Gln Val Gly Leu Ala Ala Ser Ala Leu Pro
35 40 45

acc ttc gcg cac gtg gtc atc gtg gtg gag gag aac cgc tcg cag gcc 192
Thr Phe Ala His Val Val Ile Val Val Glu Glu Asn Arg Ser Gln Ala
50 55 60

gcc atc atc ggt aac aag tcg gct ccc ttc atc aat tcg ctg gcc gcc 240
Ala Ile Ile Gly Asn Lys Ser Ala Pro Phe Ile Asn Ser Leu Ala Ala
65 70 75 80

aac ggc gcg atg atg gcc cag gcg ttc gcc gaa aca cac ccg agc gaa 288
Asn Gly Ala Met Met Ala Gln Ala Phe Ala Glu Thr His Pro Ser Glu
85 90 95

ccg aac tac ctg gca ctg ttc gct ggc aac aca ttc ggg ttg acg aag 336
Pro Asn Tyr Leu Ala Leu Phe Ala Gly Asn Thr Phe Gly Leu Thr Lys
100 105 110

aac acc tgc ccc gtc aac ggc ggc gcg ctg ccc aac ctg ggt tct gag 384
Asn Thr Cys Pro Val Asn Gly Gly Ala Leu Pro Asn Leu Gly Ser Glu
115 120 125

ttg ctc agc gcc ggt tac aca ttc atg ggg ttc gcc gaa gac ttg cct 432

Leu Leu Ser Ala Gly Tyr Thr Phe Met Gly Phe Ala Glu Asp Leu Pro
 130 135 140
 gcg gtc ggc tcc acg gtg tgc agt gcg ggc aaa tac gca cgc aaa cac 480
 Ala Val Gly Ser Thr Val Cys Ser Ala Gly Lys Tyr Ala Arg Lys His
 145 150 155 160
 gtg ccg tgg gtc aac ttc agt aac gtg ccg gcg aca ctg tcg gtg ccg 528
 Val Pro Trp Val Asn Phe Ser Asn Val Pro Ala Thr Leu Ser Val Pro
 165 170 175
 ttt tcg gca ttt ccg aag ccg cag aat tac ccc ggc ctg ccg acg gtg 576
 Phe Ser Ala Phe Pro Lys Pro Gln Asn Tyr Pro Gly Leu Pro Thr Val
 180 185 190
 tcg ttt gtc atc cct aac gcc gac aac gac atg cac gac ggc tcg atc 624
 Ser Phe Val Ile Pro Asn Ala Asp Asn Asp Met His Asp Gly Ser Ile
 195 200 205
 gcc caa ggc gac gcc tgg ctg aac cgc cac ctg tcg gca tat gcc aac 672
 Ala Gln Gly Asp Ala Trp Leu Asn Arg His Leu Ser Ala Tyr Ala Asn
 210 215 220
 tgg gcc aag aca aac aac agc ctg ctc gtt gtg acc tgg gac gaa gac 720
 Trp Ala Lys Thr Asn Asn Ser Leu Leu Val Val Thr Trp Asp Glu Asp
 225 230 235 240
 gac ggc agc agc cgc aat cag atc ccg acg gtg ttc tac ggc gcg cac 768
 Asp Gly Ser Ser Arg Asn Gln Ile Pro Thr Val Phe Tyr Gly Ala His
 245 250 255
 gtg cgg ccc gga act tac aac gag acc atc agc cac tac aac gtg ctg 816
 Val Arg Pro Gly Thr Tyr Asn Glu Thr Ile Ser His Tyr Asn Val Leu
 260 265 270
 tcc aca ttg gag cag atc tac gga ctg ccc aag acg ggt tat gcg acc 864
 Ser Thr Leu Glu Gln Ile Tyr Gly Leu Pro Lys Thr Gly Tyr Ala Thr
 275 280 285
 aat gct ccg cca ata acc gat att tgg ggc gac tag 900
 Asn Ala Pro Pro Ile Thr Asp Ile Trp Gly Asp
 290 295

<210> 12

<211> 299

<212> PRT

<213> Mycobacterium bovis

<400> 12

Met Leu Arg Gly Ile Gln Ala Leu Ser Arg Pro Leu Thr Arg Val Tyr
 1 5 10 15

Arg Ala Leu Ala Val Ile Gly Val Leu Ala Ala Ser Leu Leu Ala Ser
 20 25 30

Trp Val Gly Ala Val Pro Gln Val Gly Leu Ala Ala Ser Ala Leu Pro
 35 40 45

Thr Phe Ala His Val Val Ile Val Val Glu Glu Asn Arg Ser Gln Ala
50 55 60

Ala Ile Ile Gly Asn Lys Ser Ala Pro Phe Ile Asn Ser Leu Ala Ala
65 70 75 80

Asn Gly Ala Met Met Ala Gln Ala Phe Ala Glu Thr His Pro Ser Glu
85 90 95

Pro Asn Tyr Leu Ala Leu Phe Ala Gly Asn Thr Phe Gly Leu Thr Lys
100 105 110

Asn Thr Cys Pro Val Asn Gly Gly Ala Leu Pro Asn Leu Gly Ser Glu
115 120 125

Leu Leu Ser Ala Gly Tyr Thr Phe Met Gly Phe Ala Glu Asp Leu Pro
130 135 140

Ala Val Gly Ser Thr Val Cys Ser Ala Gly Lys Tyr Ala Arg Lys His
145 150 155 160

Val Pro Trp Val Asn Phe Ser Asn Val Pro Ala Thr Leu Ser Val Pro
165 170 175

Phe Ser Ala Phe Pro Lys Pro Gln Asn Tyr Pro Gly Leu Pro Thr Val
180 185 190

Ser Phe Val Ile Pro Asn Ala Asp Asn Asp Met His Asp Gly Ser Ile
195 200 205

Ala Gln Gly Asp Ala Trp Leu Asn Arg His Leu Ser Ala Tyr Ala Asn
210 215 220

Trp Ala Lys Thr Asn Asn Ser Leu Leu Val Val Thr Trp Asp Glu Asp
225 230 235 240

Asp Gly Ser Ser Arg Asn Gln Ile Pro Thr Val Phe Tyr Gly Ala His
245 250 255

Val Arg Pro Gly Thr Tyr Asn Glu Thr Ile Ser His Tyr Asn Val Leu
260 265 270

Ser Thr Leu Glu Gln Ile Tyr Gly Leu Pro Lys Thr Gly Tyr Ala Thr
275 280 285

Asn Ala Pro Pro Ile Thr Asp Ile Trp Gly Asp
290 295

<210> 13

<211> 903

<212> DNA

<213> Mycobacterium avium

<220>

<221> CDS

<222> (1) .. (903)

<400> 13

gtg tgc cgc gaa aat cga agt cgc aga agg ctg atc ggc ggc gca tac	48
Met Ser Arg Glu Asn Arg Ser Arg Arg Arg Leu Ile Gly Gly Ala Tyr	
1 5 10 15	
cga agc ctg cgg ctg ctc ggc gcc gtg gcc gcg gtg gcg ctg gcg gcc	96
Arg Ser Leu Arg Leu Leu Gly Ala Val Ala Ala Val Ala Leu Ala Ala	
20 25 30	
agc ccg ttg aca ccg cgc acc agc ctt gcg gca gcg gcc att ccg caa	144
Ser Pro Leu Thr Pro Arg Thr Ser Leu Ala Ala Ala Ile Pro Gln	
35 40 45	
ccg tgc cac atc gtg atc gtg gtg gag gaa aac cgt tcc gag agc ggc	192
Pro Ser His Ile Val Ile Val Val Glu Glu Asn Arg Ser Glu Ser Gly	
50 55 60	
atc atc ggc aac aag tgc gcg ccc ttc atc acc gcg ctg gcc gcg tcc	240
Ile Ile Gly Asn Lys Ser Ala Pro Phe Ile Thr Ala Leu Ala Ala Ser	
65 70 75 80	
ggc gcc aac atg acc cag tgc ttc gcc gaa acc cac ccc agc gag ccc	288
Gly Ala Asn Met Thr Gln Ser Phe Ala Glu Thr His Pro Ser Glu Pro	
85 90 95	
aat tac ctg gcg ctg ttc gcc ggc aac acg ttc ggg gtg acc aag gac	336
Asn Tyr Leu Ala Leu Phe Ala Gly Asn Thr Phe Gly Val Thr Lys Asp	
100 105 110	
ctg tgc ccg gtc aac gcc ggc gcc gca ccc aac ctg ggg tcc gaa ttg	384
Leu Cys Pro Val Asn Ala Gly Ala Ala Pro Asn Leu Gly Ser Glu Leu	
115 120 125	
ctc gcc gcc ggt tac aca ttc gcc ggc tac gcc gag ggc ctg ccg tcc	432
Leu Ala Ala Gly Tyr Thr Phe Ala Gly Tyr Ala Glu Gly Leu Pro Ser	
130 135 140	
ccg ggc tca ccg gtg tgc agt gcg ggc aag tac gcg cga aaa cat gtg	480
Pro Gly Ser Pro Val Cys Ser Ala Gly Lys Tyr Ala Arg Lys His Val	
145 150 155 160	
ccg tgg gcc aac ttc acc aac gtg ccg gcg gcg agc tgc ctg ccg ttc	528
Pro Trp Ala Asn Phe Thr Asn Val Pro Ala Ala Ser Ser Leu Pro Phe	
165 170 175	
tgc gcg ttc ccg atg ggc aac tac gcc agc ctg ccg acg gtg tgc ttc	576
Ser Ala Phe Pro Met Gly Asn Tyr Ala Ser Leu Pro Thr Val Ser Phe	
180 185 190	
gtc atc ccg aac aac gac aac aac atg cac gac ggc tgc atc gcg cag	624
Val Ile Pro Asn Asn Asp Asn Asn Met His Asp Gly Ser Ile Ala Gln	
195 200 205	
gcc gac gcc tgg ctg aac cgg cag ctg tcc ggc tac gcc aat tgg gcg	672

Ala Asp Ala Trp Leu Asn Arg Gln Leu Ser Gly Tyr Ala Asn Trp Ala
 210 215 220

ctg gcc aac aac agc ctg ctg atc gtc acc ttc gac gag gac gac aac 720
 Leu Ala Asn Asn Ser Leu Leu Ile Val Thr Phe Asp Glu Asp Asp Asn
 225 230 235 240

tcc aac gtc gga gcc agc cgc aac cag atc ccc acg gtg ttc tac ggc 768
 Ser Asn Val Gly Ala Ser Arg Asn Gln Ile Pro Thr Val Phe Tyr Gly
 245 250 255

gcc cac gtc cgc ccc ggc aac tac gcc gag cag atc aac cac tac aac 816
 Ala His Val Arg Pro Gly Asn Tyr Ala Glu Gln Ile Asn His Tyr Asn
 260 265 270

gtg ctt gcc acc ctc gag cag atg tac ggg ctg ccc aag acg ggc tat 864
 Val Leu Ala Thr Leu Glu Gln Met Tyr Gly Leu Pro Lys Thr Gly Tyr
 275 280 285

gcc gcc ggc gcc gcc ccc atc acc gac atc tgg ggc tga 903
 Ala Ala Gly Ala Ala Pro Ile Thr Asp Ile Trp Gly
 290 295 300

<210> 14

<211> 300

<212> PRT

<213> Mycobacterium avium

<400> 14

Met Ser Arg Glu Asn Arg Ser Arg Arg Arg Leu Ile Gly Gly Ala Tyr
 1 5 10 15

Arg Ser Leu Arg Leu Leu Gly Ala Val Ala Ala Val Ala Leu Ala Ala
 20 25 30

Ser Pro Leu Thr Pro Arg Thr Ser Leu Ala Ala Ala Ala Ile Pro Gln
 35 40 45

Pro Ser His Ile Val Ile Val Val Glu Glu Asn Arg Ser Glu Ser Gly
 50 55 60

Ile Ile Gly Asn Lys Ser Ala Pro Phe Ile Thr Ala Leu Ala Ala Ser
 65 70 75 80

Gly Ala Asn Met Thr Gln Ser Phe Ala Glu Thr His Pro Ser Glu Pro
 85 90 95

Asn Tyr Leu Ala Leu Phe Ala Gly Asn Thr Phe Gly Val Thr Lys Asp
 100 105 110

Leu Cys Pro Val Asn Ala Gly Ala Ala Pro Asn Leu Gly Ser Glu Leu
 115 120 125

Leu Ala Ala Gly Tyr Thr Phe Ala Gly Tyr Ala Glu Gly Leu Pro Ser
130 135 140

Pro Gly Ser Pro Val Cys Ser Ala Gly Lys Tyr Ala Arg Lys His Val
145 150 155 160

Pro Trp Ala Asn Phe Thr Asn Val Pro Ala Ala Ser Ser Leu Pro Phe
165 170 175

Ser Ala Phe Pro Met Gly Asn Tyr Ala Ser Leu Pro Thr Val Ser Phe
180 185 190

Val Ile Pro Asn Asn Asp Asn Asn Met His Asp Gly Ser Ile Ala Gln
195 200 205

Ala Asp Ala Trp Leu Asn Arg Gln Leu Ser Gly Tyr Ala Asn Trp Ala
210 215 220

Leu Ala Asn Asn Ser Leu Leu Ile Val Thr Phe Asp Glu Asp Asp Asn
225 230 235 240

Ser Asn Val Gly Ala Ser Arg Asn Gln Ile Pro Thr Val Phe Tyr Gly
245 250 255

Ala His Val Arg Pro Gly Asn Tyr Ala Glu Gln Ile Asn His Tyr Asn
260 265 270

Val Leu Ala Thr Leu Glu Gln Met Tyr Gly Leu Pro Lys Thr Gly Tyr
275 280 285

Ala Ala Gly Ala Ala Pro Ile Thr Asp Ile Trp Gly
290 295 300

<210> 15

<211> 888

<212> DNA

<213> Mycobacterium marinum

<220>

<221> CDS

<222> (1)..(888)

<400> 15

gtg tgt ggc ctg aaa cag cgt ttt acc agt aca ttt cga gct ctg gcg 48
Met Cys Gly Leu Lys Gln Arg Phe Thr Ser Thr Phe Arg Ala Leu Ala
1 5 10 15

gta ctc ggc gcg gtg gcg gta tcc cta ccg gcc cac ggt agc gac gct 96

Val	Leu	Gly	Ala	Val	Ala	Val	Ser	Leu	Pro	Ala	His	Gly	Ser	Asp	Ala	
			20					25					30			
ccc	ccg	cgt	atc	gac	ctg	acc	gcc	act	gcg	ttg	ccg	gcg	ttc	tca	cat	144
Pro	Pro	Arg	Ile	Asp	Leu	Thr	Ala	Thr	Ala	Leu	Pro	Ala	Phe	Ser	His	
		35					40					45				
gtg	gtg	gtc	gtg	gtg	gag	gag	aac	cat	tcg	cag	gcc	aac	atc	att	ggc	192
Val	Val	Val	Val	Val	Glu	Glu	Asn	His	Ser	Gln	Ala	Asn	Ile	Ile	Gly	
	50					55					60					
aac	aag	gcg	gcc	ccg	ttc	atc	aat	gcg	ctg	gcc	gcc	aat	ggc	gcg	atg	240
Asn	Lys	Ala	Ala	Pro	Phe	Ile	Asn	Ala	Leu	Ala	Ala	Asn	Gly	Ala	Met	
65					70				75						80	
atg	tcg	cag	tcg	ttc	gcc	gaa	acg	cac	ccc	agc	gaa	ccc	aac	tac	ctg	288
Met	Ser	Gln	Ser	Phe	Ala	Glu	Thr	His	Pro	Ser	Glu	Pro	Asn	Tyr	Leu	
				85					90					95		
gcc	ttg	ttc	gcc	ggt	acc	acc	ttc	ggc	ttg	aag	aag	aac	acg	tgt	ccg	336
Ala	Leu	Phe	Ala	Gly	Thr	Thr	Phe	Gly	Leu	Lys	Lys	Asn	Thr	Cys	Pro	
			100					105					110			
gtc	aat	gcg	ggc	agc	acg	ccc	aac	ctg	gct	tcg	gag	ttg	ctc	gcc	gcg	384
Val	Asn	Ala	Gly	Ser	Thr	Pro	Asn	Leu	Ala	Ser	Glu	Leu	Leu	Ala	Ala	
		115					120					125				
ggc	cac	acg	ttc	gta	ggt	ttc	gcc	gag	agc	ctg	ccc	gaa	gtc	ggt	tcg	432
Gly	His	Thr	Phe	Val	Gly	Phe	Ala	Glu	Ser	Leu	Pro	Glu	Val	Gly	Ser	
	130					135					140					
acg	gtc	tgc	agc	gcc	gga	aag	tac	ggg	cgc	aag	cat	gcg	cct	tgg	gtg	480
Thr	Val	Cys	Ser	Ala	Gly	Lys	Tyr	Gly	Arg	Lys	His	Ala	Pro	Trp	Val	
145					150				155						160	
aac	ttc	agc	aat	gtt	ccg	gcc	acg	ctg	tcg	atg	ccc	ttc	tcc	gcg	ttt	528
Asn	Phe	Ser	Asn	Val	Pro	Ala	Thr	Leu	Ser	Met	Pro	Phe	Ser	Ala	Phe	
				165					170					175		
ccg	acg	ccg	gcg	gac	tac	gcc	agg	ctg	ccc	acg	gtg	tcc	ttc	gtc	atc	576
Pro	Thr	Pro	Ala	Asp	Tyr	Ala	Arg	Leu	Pro	Thr	Val	Ser	Phe	Val	Ile	
			180					185					190			
ccc	aac	ggg	gat	aac	aac	atg	cac	gac	ggc	acc	atc	gcg	gca	gct	gac	624
Pro	Asn	Gly	Asp	Asn	Asn	Met	His	Asp	Gly	Thr	Ile	Ala	Ala	Ala	Asp	
		195					200					205				
gag	tgg	ttg	aac	cgt	caa	ctg	tcg	ccg	tac	gcc	aac	tgg	gcc	cga	tcc	672
Glu	Trp	Leu	Asn	Arg	Gln	Leu	Ser	Pro	Tyr	Ala	Asn	Trp	Ala	Arg	Ser	
	210					215						220				
aac	aac	agc	ctg	ctg	atc	gtg	acg	tgg	gat	gag	gac	gac	ggc	ggc	agc	720
Asn	Asn	Ser	Leu	Leu	Ile	Val	Thr	Trp	Asp	Glu	Asp	Asp	Gly	Gly	Ser	
225					230				235						240	
cgc	aac	cag	att	ccc	acg	gtg	ttc	tac	ggc	gca	cac	gta	cgg	ccg	ggc	768
Arg	Asn	Gln	Ile	Pro	Thr	Val	Phe	Tyr	Gly	Ala	His	Val	Arg	Pro	Gly	
				245				250						255		
act	tac	aac	cag	acc	atc	agc	cac	tac	aac	gtg	ctt	tcc	acg	ctg	gag	816
Thr	Tyr	Asn	Gln	Thr	Ile	Ser	His	Tyr	Asn	Val	Leu	Ser	Thr	Leu	Glu	
			260					265					270			
cag	atg	tac	ggc	ttg	ccc	aag	acg	ggt	ttc	gcg	gcg	aac	gcc	ccg	gtc	864
Gln	Met	Tyr	Gly	Leu	Pro	Lys	Thr	Gly	Phe	Ala	Ala	Asn	Ala	Pro	Val	
		275					280					285				
atc	gct	gat	atc	tgg	ggc	ggc	taa									888

Ile Ala Asp Ile Trp Gly Gly
290 295

<210> 16

<211> 295

<212> PRT

<213> Mycobacterium marinum

<400> 16

Met Cys Gly Leu Lys Gln Arg Phe Thr Ser Thr Phe Arg Ala Leu Ala
1 5 10 15

Val Leu Gly Ala Val Ala Val Ser Leu Pro Ala His Gly Ser Asp Ala
20 25 30

Pro Pro Arg Ile Asp Leu Thr Ala Thr Ala Leu Pro Ala Phe Ser His
35 40 45

Val Val Val Val Val Glu Glu Asn His Ser Gln Ala Asn Ile Ile Gly
50 55 60

Asn Lys Ala Ala Pro Phe Ile Asn Ala Leu Ala Ala Asn Gly Ala Met
65 70 75 80

Met Ser Gln Ser Phe Ala Glu Thr His Pro Ser Glu Pro Asn Tyr Leu
85 90 95

Ala Leu Phe Ala Gly Thr Thr Phe Gly Leu Lys Lys Asn Thr Cys Pro
100 105 110

Val Asn Ala Gly Ser Thr Pro Asn Leu Ala Ser Glu Leu Leu Ala Ala
115 120 125

Gly His Thr Phe Val Gly Phe Ala Glu Ser Leu Pro Glu Val Gly Ser
130 135 140

Thr Val Cys Ser Ala Gly Lys Tyr Gly Arg Lys His Ala Pro Trp Val
145 150 155 160

Asn Phe Ser Asn Val Pro Ala Thr Leu Ser Met Pro Phe Ser Ala Phe
165 170 175

Pro Thr Pro Ala Asp Tyr Ala Arg Leu Pro Thr Val Ser Phe Val Ile
180 185 190

Pro Asn Gly Asp Asn Asn Met His Asp Gly Thr Ile Ala Ala Ala Asp
195 200 205

Glu Trp Leu Asn Arg Gln Leu Ser Pro Tyr Ala Asn Trp Ala Arg Ser
 210 215 220

Asn Asn Ser Leu Leu Ile Val Thr Trp Asp Glu Asp Asp Gly Gly Ser
 225 230 235 240

Arg Asn Gln Ile Pro Thr Val Phe Tyr Gly Ala His Val Arg Pro Gly
 245 250 255

Thr Tyr Asn Gln Thr Ile Ser His Tyr Asn Val Leu Ser Thr Leu Glu
 260 265 270

Gln Met Tyr Gly Leu Pro Lys Thr Gly Phe Ala Ala Asn Ala Pro Val
 275 280 285

Ile Ala Asp Ile Trp Gly Gly
 290 295

<210> 17

<211> 1291

<212> DNA

<213> *Penicillium chrysogenum*

<220>

<221> CDS

<222> (1) .. (213)

<220>

<221> intro

<222> (217) .. (244)

<220>

<221> CDS

<222> (245) .. (1288)

<400> 17

atg ctc acc aaa caa acc ctt ctc gcg ttc gtc ggg gcc ctc gcc ctc 48
 Met Leu Thr Lys Gln Thr Leu Leu Ala Phe Val Gly Ala Leu Ala Leu
 1 5 10 15

gcc acg ggt aca act acc act gaa gag acc cca act cag gct gag att 96
 Ala Thr Gly Thr Thr Thr Thr Glu Glu Thr Pro Thr Gln Ala Glu Ile
 20 25 30

gat gca gca cgt gct acg gcc ctg cct tac tct cct gtg tca aac gta 144

Asp	Ala	Ala	Arg	Ala	Thr	Ala	Leu	Pro	Tyr	Ser	Pro	Val	Ser	Asn	Val		
	35						40					45					
aag	ggg	ttg	gcc	ttt	gat	cgt	ttc	gtg	aac	atc	tgg	ctc	gag	aac	aca		192
Lys	Gly	Leu	Ala	Phe	Asp	Arg	Phe	Val	Asn	Ile	Trp	Leu	Glu	Asn	Thr		
	50					55					60						
gta	ggg	ttc	ccg	ttg	aat	ata	taacaatgac	cacgcgctca	cccccttgta	g							244
Val	Gly	Phe	Pro	Leu	Asn	Ile											
	65				70												
gac	ttt	gaa	ccc	gct	gct	tta	gac	gag	aac	ctg	tcc	aag	ctg	gcc	aag		292
Asp	Phe	Glu	Pro	Ala	Ala	Leu	Asp	Glu	Asn	Leu	Ser	Lys	Leu	Ala	Lys		
			75					80					85				
gag	ggg	atc	ctc	ctg	acc	aac	tac	ttt	gcc	atc	tct	cac	ccc	tcg	cag		340
Glu	Gly	Ile	Leu	Leu	Thr	Asn	Tyr	Phe	Ala	Ile	Ser	His	Pro	Ser	Gln		
		90					95					100					
ccc	aac	tac	tgt	gct	tcc	gcc	ggg	ggg	gac	aca	ttc	ggc	atg	gat	aat		388
Pro	Asn	Tyr	Cys	Ala	Ser	Ala	Gly	Gly	Asp	Thr	Phe	Gly	Met	Asp	Asn		
	105					110					115						
gac	gac	ttc	cta	caa	atc	cct	tcg	aat	gtc	tca	act	att	gcc	gat	ctc		436
Asp	Asp	Phe	Leu	Gln	Ile	Pro	Ser	Asn	Val	Ser	Thr	Ile	Ala	Asp	Leu		
	120				125				130						135		
ttt	gat	act	aag	cac	atc	tct	tgg	ggg	gaa	tac	caa	gaa	gac	atg	ccc		484
Phe	Asp	Thr	Lys	His	Ile	Ser	Trp	Gly	Glu	Tyr	Gln	Glu	Asp	Met	Pro		
				140				145						150			
tat	gct	ggc	tac	caa	ggc	aaa	cgg	tat	ccc	ctc	agc	ggg	ccg	aac	cag		532
Tyr	Ala	Gly	Tyr	Gln	Gly	Lys	Arg	Tyr	Pro	Leu	Ser	Gly	Pro	Asn	Gln		
		155						160					165				
tac	gtg	cgc	aag	cac	aac	ccg	ctg	ggt	ttg	ttt	aac	tcg	ggt	acc	gac		580
Tyr	Val	Arg	Lys	His	Asn	Pro	Leu	Val	Leu	Phe	Asn	Ser	Val	Thr	Asp		
		170					175					180					
gac	gcc	gtg	cgc	ccg	cgc	caa	atc	aag	aat	ttc	acc	act	ttc	tac	gac		628
Asp	Ala	Val	Arg	Pro	Arg	Gln	Ile	Lys	Asn	Phe	Thr	Thr	Phe	Tyr	Asp		
		185				190					195						
gat	ctg	aag	cac	cac	agc	ctt	ccc	caa	cac	atg	ttc	atc	aca	ccg	aac		676
Asp	Leu	Lys	His	His	Ser	Leu	Pro	Gln	His	Met	Phe	Ile	Thr	Pro	Asn		
	200				205					210					215		
atg	acc	aat	gac	gcc	cac	gac	acg	aac	atc	act	gtg	gcc	ggg	aac	tgg		724
Met	Thr	Asn	Asp	Ala	His	Asp	Thr	Asn	Ile	Thr	Val	Ala	Gly	Asn	Trp		
				220				225						230			
gtc	gat	cgc	ttc	ctg	tct	cct	cta	ctg	aag	aac	gag	tac	ttc	acc	aag		772
Val	Asp	Arg	Phe	Leu	Ser	Pro	Leu	Leu	Lys	Asn	Glu	Tyr	Phe	Thr	Lys		
			235					240					245				
gac	agc	cta	gtg	cta	ctc	acc	ttt	gac	gag	gga	gac	acc	tac	tcc	tac		820
Asp	Ser	Leu	Val	Leu	Leu	Thr	Phe	Asp	Glu	Gly	Asp	Thr	Tyr	Ser	Tyr		
		250					255					260					
ccc	aac	cgg	gtc	ttc	agc	ttc	ctt	ggt	gga	ggg	gct	atc	cca	gag	cac		868
Pro	Asn	Arg	Val	Phe	Ser	Phe	Leu	Val	Gly	Gly	Ala	Ile	Pro	Glu	His		
		265				270					275						
ctg	aag	ggg	acc	act	gac	gac	act	ttc	tac	acc	cac	tac	tca	att	gtc		916
Leu	Lys	Gly	Thr	Thr	Asp	Asp	Thr	Phe	Tyr	Thr	His	Tyr	Ser	Ile	Val		
		280			285					290					295		
gct	tcc	ctg	tct	gct	aac	tgg	ggg	tta	ccc	tcg	ctt	ggg	cgc	tgg	gat		964

Ala Ser Leu Ser Ala Asn Trp Gly Leu Pro Ser Leu Gly Arg Trp Asp
 300 305 310

tgt ggc gcc aac ctg ctg aag atg gtc gct gac aag acc ggc tat gtc 1012
 Cys Gly Ala Asn Leu Leu Lys Met Val Ala Asp Lys Thr Gly Tyr Val
 315 320 325

aac tgg gaa gtt gat acc agc aat gtc tac ctc aac gag act tac cct 1060
 Asn Trp Glu Val Asp Thr Ser Asn Val Tyr Leu Asn Glu Thr Tyr Pro
 330 335 340

gga cct atg tct acc gac aac tat tcc tct aag tgg gcc gtt cct gcc 1108
 Gly Pro Met Ser Thr Asp Asn Tyr Ser Ser Lys Trp Ala Val Pro Ala
 345 350 355

acc aag ggc aaa tgc tct gct ggc cat ggc att gct gag gtc gtg aag 1156
 Thr Lys Gly Lys Cys Ser Ala Gly His Gly Ile Ala Glu Val Val Lys
 360 365 370 375

aat acc tac cac ggg ctt caa ccc acc tac gac tat gcc agc cct gta 1204
 Asn Thr Tyr His Gly Leu Gln Pro Thr Tyr Asp Tyr Ala Ser Pro Val
 380 385 390

ccg tat gac gtg acc agt gga aac aac gtc ggc atc aag tac cac cgc 1252
 Pro Tyr Asp Val Thr Ser Gly Asn Asn Val Gly Ile Lys Tyr His Arg
 395 400 405

act ctg gta tgt atc ctt tca tgt tct tcc ctt tca tga 1291
 Thr Leu Val Cys Ile Leu Ser Cys Ser Ser Leu Ser
 410 415

<210> 18

<211> 71

<212> PRT

<213> *Penicillium chrysogenum*

<400> 18

Met Leu Thr Lys Gln Thr Leu Leu Ala Phe Val Gly Ala Leu Ala Leu
 1 5 10 15

Ala Thr Gly Thr Thr Thr Thr Glu Glu Thr Pro Thr Gln Ala Glu Ile
 20 25 30

Asp Ala Ala Arg Ala Thr Ala Leu Pro Tyr Ser Pro Val Ser Asn Val
 35 40 45

Lys Gly Leu Ala Phe Asp Arg Phe Val Asn Ile Trp Leu Glu Asn Thr
 50 55 60

Val Gly Phe Pro Leu Asn Ile
 65 70

<210> 19

<211> 348

<212> PRT

<213> Penicillium chrysogenum

<400> 19

Asp Phe Glu Pro Ala Ala Leu Asp Glu Asn Leu Ser Lys Leu Ala Lys
 1 5 10 15

Glu Gly Ile Leu Leu Thr Asn Tyr Phe Ala Ile Ser His Pro Ser Gln
 20 25 30

Pro Asn Tyr Cys Ala Ser Ala Gly Gly Asp Thr Phe Gly Met Asp Asn
 35 40 45

Asp Asp Phe Leu Gln Ile Pro Ser Asn Val Ser Thr Ile Ala Asp Leu
 50 55 60

Phe Asp Thr Lys His Ile Ser Trp Gly Glu Tyr Gln Glu Asp Met Pro
 65 70 75 80

Tyr Ala Gly Tyr Gln Gly Lys Arg Tyr Pro Leu Ser Gly Pro Asn Gln
 85 90 95

Tyr Val Arg Lys His Asn Pro Leu Val Leu Phe Asn Ser Val Thr Asp
 100 105 110

Asp Ala Val Arg Pro Arg Gln Ile Lys Asn Phe Thr Thr Phe Tyr Asp
 115 120 125

Asp Leu Lys His His Ser Leu Pro Gln His Met Phe Ile Thr Pro Asn
 130 135 140

Met Thr Asn Asp Ala His Asp Thr Asn Ile Thr Val Ala Gly Asn Trp
 145 150 155 160

Val Asp Arg Phe Leu Ser Pro Leu Leu Lys Asn Glu Tyr Phe Thr Lys
 165 170 175

Asp Ser Leu Val Leu Leu Thr Phe Asp Glu Gly Asp Thr Tyr Ser Tyr
 180 185 190

Pro Asn Arg Val Phe Ser Phe Leu Val Gly Gly Ala Ile Pro Glu His
 195 200 205

Leu Lys Gly Thr Thr Asp Asp Thr Phe Tyr Thr His Tyr Ser Ile Val
 210 215 220

Ala Ser Leu Ser Ala Asn Trp Gly Leu Pro Ser Leu Gly Arg Trp Asp
 225 230 235 240

Cys Gly Ala Asn Leu Leu Lys Met Val Ala Asp Lys Thr Gly Tyr Val
 245 250 255

Asn Trp Glu Val Asp Thr Ser Asn Val Tyr Leu Asn Glu Thr Tyr Pro
 260 265 270

Gly Pro Met Ser Thr Asp Asn Tyr Ser Ser Lys Trp Ala Val Pro Ala
 275 280 285

Thr Lys Gly Lys Cys Ser Ala Gly His Gly Ile Ala Glu Val Val Lys
 290 295 300

Asn Thr Tyr His Gly Leu Gln Pro Thr Tyr Asp Tyr Ala Ser Pro Val
 305 310 315 320

Pro Tyr Asp Val Thr Ser Gly Asn Asn Val Gly Ile Lys Tyr His Arg
 325 330 335

Thr Leu Val Cys Ile Leu Ser Cys Ser Ser Leu Ser
 340 345

<210> 20

<211> 1457

<212> DNA

<213> *Aspergillus fumigatus*

<220>

<221> CDS

<222> (1) .. (186)

<220>

<221> Intron

<222> (187) .. (239)

<220>

<221> CDS

<222> (240) .. (301)

<220>

<221> Intron

<222> (302) .. (361)

<220>

<221> CDS

<222> (362)..(1454)

<400> 20

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1 5 10 15	
gct cag act gct act gag aag gag cct tcg ctg tct gcg ata gaa tct	96
Ala Gln Thr Ala Thr Glu Lys Glu Pro Ser Leu Ser Ala Ile Glu Ser	
20 25 30	
gca gca gcc tcc atc cag cct tac tct ccc gtt tcg aac gtt gag ggt	144
Ala Ala Ala Ser Ile Gln Pro Tyr Ser Pro Val Ser Asn Val Glu Gly	
35 40 45	
gtt gca ttt aat cgc ttc ttc caa gtg tgg ctt gag aat att	186
Val Ala Phe Asn Arg Phe Phe Gln Val Trp Leu Glu Asn Ile	
50 55 60	
gatatgtgac accttaccaa tcagagaatc ttgttcaaag ctgacctcag aag gat	242
Asp	
tac gag gat gct gcg gcg gat gag aac atg aaa tgg ctg gcc tcg caa	290
Tyr Glu Asp Ala Ala Ala Asp Glu Asn Met Lys Trp Leu Ala Ser Gln	
65 70 75	
ggg atc ctg ct gtaagacctc atatcggcca tctgctcacg atttgaactt	341
Gly Ile Leu Leu	
80	
ogaactaatt tactccacag c acc aat ttc tat gca gtc acg cat cct tca	392
Thr Asn Phe Tyr Ala Val Thr His Pro Ser	
85 90	
gag cca aac tac tgc gct gct gtt gga ggc gac aca ttt ggc atg gac	440
Glu Pro Asn Tyr Cys Ala Ala Val Gly Gly Asp Thr Phe Gly Met Asp	
95 100 105	
aat gac aac ttt aac cag att cct gcc aat gtt tct act gtc gct gat	488
Asn Asp Asn Phe Asn Ile Pro Ala Asn Val Ser Thr Val Ala Asp	
110 115 120 125	
ctc ctg gac acc aaa aac att gct tgg gga gag tat cag gag cac tta	536
Leu Leu Asp Thr Lys Asn Ile Ala Trp Gly Glu Tyr Gln Glu His Leu	
130 135 140	
cct tat ccc gga ttc caa ggt ttc aac tat tcc aac cag gag act tat	584
Pro Tyr Pro Gly Phe Gln Gly Phe Asn Tyr Ser Asn Gln Glu Thr Tyr	
145 150 155	
gtc aat gac tat gtg cgc aag cat aac cca ctg gtc ttg tat gac tct	632
Val Asn Asp Tyr Val Arg Lys His Asn Pro Leu Val Leu Tyr Asp Ser	
160 165 170	
gtc acc aag aac agc act cgt ttg cgc cag atc aag aac ttt acc agc	680
Val Thr Lys Asn Ser Thr Arg Leu Arg Gln Ile Lys Asn Phe Thr Ser	
175 180 185	
ttc gag gac gac ctg gcc aac aag aaa ctt cct caa tgg gca ttt atc	728

Phe 190	Glu	Asp	Asp	Leu	Ala 195	Asn	Lys	Lys	Leu	Pro 200	Gln	Trp	Ala	Phe	Ile 205	
act	cca	aac	atg	acc	aac	gac	gct	cat	gac	acc	aac	att	act	ttc	gga	776
Thr	Pro	Asn	Met	Thr	Asn	Asp	Ala	His	Asp	Thr	Asn	Ile	Thr	Phe	Gly	
				210					215					220		
gcc	aaa	tgg	gag	cga	agc	tgg	att	gcg	ccc	ttg	ctc	aac	aac	tca	tac	824
Ala	Lys	Trp	Glu	Arg	Ser	Trp	Ile	Ala	Pro	Leu	Leu	Asn	Asn	Ser	Tyr	
			225					230					235			
ttc	atg	aat	gat	acc	cta	atc	cta	ctt	acc	ttt	gat	gag	gat	ggc	act	872
Phe	Met	Asn	Asp	Thr	Leu	Ile	Leu	Leu	Thr	Phe	Asp	Glu	Asp	Gly	Thr	
		240					245					250				
tat	tcc	aag	agc	aac	aag	atc	ttc	agt	gtt	ctt	ctc	ggt	ggt	gcc	att	920
Tyr	Ser	Lys	Ser	Asn	Lys	Ile	Phe	Ser	Val	Leu	Leu	Gly	Gly	Ala	Ile	
		255				260					265					
ccc	gat	gag	ctg	aag	ggt	act	cag	gac	gat	acg	ttc	tat	acc	cac	tac	968
Pro	Asp	Glu	Leu	Lys	Gly	Thr	Gln	Asp	Asp	Thr	Phe	Tyr	Thr	His	Tyr	
270					275					280					285	
tca	gtg	att	gcg	tcc	gtg	tcc	gcg	aac	tgg	ggc	ctt	cct	tcg	ttg	gga	1016
Ser	Val	Ile	Ala	Ser	Val	Ser	Ala	Asn	Trp	Gly	Leu	Pro	Ser	Leu	Gly	
				290					295					300		
agg	tgg	gat	tgt	ggt	gcg	aac	att	ctt	gag	att	gtg	gca	aac	aag	acg	1064
Arg	Trp	Asp	Cys	Gly	Ala	Asn	Ile	Leu	Glu	Ile	Val	Ala	Asn	Lys	Thr	
			305					310					315			
gga	tat	gtc	aac	tac	gac	gtt	gac	aca	acc	aat	ctc	cgc	ctc	aac	gag	1112
Gly	Tyr	Val	Asn	Tyr	Asp	Val	Asp	Thr	Thr	Asn	Leu	Arg	Leu	Asn	Glu	
		320					325					330				
acc	tac	ccc	ggt	ccc	atg	tca	gcg	ggc	gaa	tac	tcg	aaa	tac	tcc	cct	1160
Thr	Tyr	Pro	Gly	Pro	Met	Ser	Ala	Gly	Glu	Tyr	Ser	Lys	Tyr	Ser	Pro	
		335				340					345					
gtc	tgg	ccg	aat	gcc	ttg	acc	cgt	ggt	gac	tgc	tct	gct	ggc	cat	ggc	1208
Val	Trp	Pro	Asn	Ala	Leu	Thr	Arg	Gly	Asp	Cys	Ser	Ala	Gly	His	Gly	
350					355					360					365	
att	ttg	gac	att	gtc	aag	gag	acc	tac	gcc	aac	acg	gag	cca	aca	tac	1256
Ile	Leu	Asp	Ile	Val	Lys	Glu	Thr	Tyr	Ala	Asn	Thr	Glu	Pro	Thr	Tyr	
				370					375					380		
aac	tat	tcg	agc	ccc	ttc	cca	tat	gac	act	gcg	agc	aac	tac	aac	acc	1304
Asn	Tyr	Ser	Ser	Pro	Phe	Pro	Tyr	Asp	Thr	Ala	Ser	Asn	Tyr	Asn	Thr	
			385					390					395			
aag	gtg	act	gcc	acc	aaa	aag	aat	gtc	acc	ggt	aca	cat	aga	agt	tct	1352
Lys	Val	Thr	Ala	Thr	Lys	Lys	Asn	Val	Thr	Gly	Thr	His	Arg	Ser	Ser	
		400					405					410				
tct	tcc	tcc	tct	ccg	tca	gct	agc	tcc	aac	gcc	gct	gtt	tct	gct	gtc	1400
Ser	Ser	Ser	Ser	Pro	Ser	Ala	Ser	Ser	Asn	Ala	Ala	Val	Ser	Ala	Val	
		415				420					425					
gct	cct	gca	gcc	ggt	gtc	tct	ggt	ctc	ctc	ttg	gga	ctc	gct	cta	aac	1448
Ala	Pro	Ala	Ala	Gly	Val	Ser	Gly	Leu	Leu	Leu	Gly	Leu	Ala	Leu	Asn	
430					435					440					445	
ctg	ctt	taa														1457
Leu	Leu															

<210> 21

<211> 447

<212> PRT

<213> *Aspergillus fumigatus*

<400> 21

Met Lys Pro Ser Val Ala Thr Leu Leu Ala Thr Val Ser Leu Val Tyr
 1 5 10 15

Ala Gln Thr Ala Thr Glu Lys Glu Pro Ser Leu Ser Ala Ile Glu Ser
 20 25 30

Ala Ala Ala Ser Ile Gln Pro Tyr Ser Pro Val Ser Asn Val Glu Gly
 35 40 45

Val Ala Phe Asn Arg Phe Phe Gln Val Trp Leu Glu Asn Ile Asp Tyr
 50 55 60

Glu Asp Ala Ala Ala Asp Glu Asn Met Lys Trp Leu Ala Ser Gln Gly
 65 70 75 80

Ile Leu Leu Thr Asn Phe Tyr Ala Val Thr His Pro Ser Glu Pro Asn
 85 90 95

Tyr Cys Ala Ala Val Gly Gly Asp Thr Phe Gly Met Asp Asn Asp Asn
 100 105 110

Phe Asn Gln Ile Pro Ala Asn Val Ser Thr Val Ala Asp Leu Leu Asp
 115 120 125

Thr Lys Asn Ile Ala Trp Gly Glu Tyr Gln Glu His Leu Pro Tyr Pro
 130 135 140

Gly Phe Gln Gly Phe Asn Tyr Ser Asn Gln Glu Thr Tyr Val Asn Asp
 145 150 155 160

Tyr Val Arg Lys His Asn Pro Leu Val Leu Tyr Asp Ser Val Thr Lys
 165 170 175

Asn Ser Thr Arg Leu Arg Gln Ile Lys Asn Phe Thr Ser Phe Glu Asp
 180 185 190

Asp Leu Ala Asn Lys Lys Leu Pro Gln Trp Ala Phe Ile Thr Pro Asn
 195 200 205

Met Thr Asn Asp Ala His Asp Thr Asn Ile Thr Phe Gly Ala Lys Trp
 210 215 220

Glu Arg Ser Trp Ile Ala Pro Leu Leu Asn Asn Ser Tyr Phe Met Asn
225 230 235 240

Asp Thr Leu Ile Leu Leu Thr Phe Asp Glu Asp Gly Thr Tyr Ser Lys
245 250 255

Ser Asn Lys Ile Phe Ser Val Leu Leu Gly Gly Ala Ile Pro Asp Glu
260 265 270

Leu Lys Gly Thr Gln Asp Asp Thr Phe Tyr Thr His Tyr Ser Val Ile
275 280 285

Ala Ser Val Ser Ala Asn Trp Gly Leu Pro Ser Leu Gly Arg Trp Asp
290 295 300

Cys Gly Ala Asn Ile Leu Glu Ile Val Ala Asn Lys Thr Gly Tyr Val
305 310 315 320

Asn Tyr Asp Val Asp Thr Thr Asn Leu Arg Leu Asn Glu Thr Tyr Pro
325 330 335

Gly Pro Met Ser Ala Gly Glu Tyr Ser Lys Tyr Ser Pro Val Trp Pro
340 345 350

Asn Ala Leu Thr Arg Gly Asp Cys Ser Ala Gly His Gly Ile Leu Asp
355 360 365

Ile Val Lys Glu Thr Tyr Ala Asn Thr Glu Pro Thr Tyr Asn Tyr Ser
370 375 380

Ser Pro Phe Pro Tyr Asp Thr Ala Ser Asn Tyr Asn Thr Lys Val Thr
385 390 395 400

Ala Thr Lys Lys Asn Val Thr Gly Thr His Arg Ser Ser Ser Ser Ser
405 410 415

Ser Pro Ser Ala Ser Ser Asn Ala Ala Val Ser Ala Val Ala Pro Ala
420 425 430

Ala Gly Val Ser Gly Leu Leu Leu Gly Leu Ala Leu Asn Leu Leu
435 440 445

<210> 22

<211> 8

<212> PRT

<213> Mycobacterium tuberculosis

<400> 22

Asn Asp Met His Asp Gly Ser Ile
1 5

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